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The Phytoremediation of Heavy Metal Contaminated Roadside Soils in Libya by Eucalyptus Camaldeulensis

Sallami, Khaled

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The Phytoremediation of Heavy Metal Contaminated Roadside Soils in Libya by *Eucalyptus Camaldeulensis*

Khaled Sallami

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Declaration

Except where specific reference is made to other sources, the work presented here is the original work of the author. It has not been submitted, in part or in whole, for any other degree.

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Acronyms

ANOVA	Analysis of Variance
CEC	Cation exchange capacity
GC	Greenwaste Compost
DTPA	Diethylenetrinitrilo-pentaacetic acid
DW	Dry weight
E. camaldeulensis	Eucalyptus camaldeulensis
EC	Condectivity
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylebis[oxyethylenetrinitrilo]-tetraacetic acid
HEDTA	Hydroxyethyl-ethylene-dinitrilo-triacetic acid
M. sativum	Medicago sativum
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
WHC	Water holding capacity
OM	Orginic matter
MC	Moiture contant
BD	Bulk density
FPR	Fresh plant root
FPS	Fresh plant shoot
DRW	Dry root weight
DSW	Dry shoot weight
BCF	Bio-concentration factor
TF	Translocation factor
PCs	Phytochelatins

Abstract

There is a public concern over the potential accumulation of heavy metals in soils. Numerous studies have already demonstrated that areas in close proximity to vehicular traffic are marked noticeably by contamination of soil, air and water. Hence, such activities can affect humans and other living organisms. The aim of this study is to investigate the pollution of soils caused by vehicular traffic, on agricultural land in Azzawiyah, Liby with the view of assessing potential application of phytoremediation options for the remediation of contaminated soils and determine whether soil amendments would improve soil remediation.

In an effort to improve the status of pollution of soils by vehicular traffic, a phytoremediation method of remediation of contaminated land has been used in this study, as it is relatively inexpensive and has the potential through the appropriate selection of plant species to be effective. This method is a soil clean up technology that uses the ability of metal accumulator plants to extract metal from contaminated soil with their roots and to concentrate these metals in above-ground plant parts.

In this study, the investigation area was in Azzawiyah city where the soil samples and *Doedonea viscose* plant were collected from the road side. These soil samples were analysed using different experiments to determine physical and chemical properties, such as pH, OM and CEC. Heavy metals in soil and *Doedonea viscose* shoot and root were analysed using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

The findings of the study show that all soils samples collected along the highway road connecting Azzawiyah with the southern parts of Libya were found to be granular with a sandy texture. It is also found that the metal content in soil collected from the site, which is close to the roadside was relatively higher than that soil collected from the agricultural field in the same area. Furthermore, the level of Pb (840mg/kg^{-1}) in roadside soils was higher than the natural levels of Pb in soils. In addition, *Doedonea viscose* plant was not a hyperaccumulor plant.

Greenhouse experiments used three plants (*E. camaldeulensis, Brassica Juncea* and *Medicago sativum*) to uptake heavy metal, such as Cd, Zn and Pb from the soil samples. The greenhouse experiment results indicate that *E. camaldeulensis* was the best plant species for phytoremediation of Pb contaminated soils than the other two plants species (*Brassica Juncea, Medicago sativum*).

The efficiency of the *E. camaldeulensis* was increased by adding amendments (e.g. compost, compost, EDTA, Hoagland solution and *Alcaligenes eutrophus*) to the plants pots in order to uptake the lead form soil samples. The results of the pots amendments experiments indicate that 15 mmol of EDTA and bacterial inoculums (*Alcaligenes eutrophus*) were the best amendments to extract lead from the soils. The study suggests that using the *Alcaligenes eutrophus* with the *E. camaldeulensis* are more suitable for phytoremediation in terms of accumulation and cost.

Published Papers

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Chapter 1

Introduction

1.1. Introduction

In modern economies, several types of activity, including transportation, industry and agriculture, are the main causes of soil pollution. As a result, the productivity potential of soils may be reduced. Plants grown in contaminated soils may absorb toxic materials that eventually cause problems in the food chain (Anwar, 2003). Soils are also considered as a sink for trapping trace elements; hence, they play an important role in environmental cycle of these elements (Webber, 1981). Some heavy metals (e.g. Cu and Zn) are micronutrients essential for plant growth and, therefore, are useful to the crops. However, their availability in excess can hinder growth or even become toxic to plants. Heavy metals (e.g. As, Cd, Pb and Hg) which are not essential for plant or animal nutrition may be toxic to animals and humans above defined levels (Haque and Subramanian, 1982; Alloway and Ayres, 1997; Kamnev and Van Der Lelie, 2000; Caselles *et al.*, 2001and Adriano *et al.* 2004).

Many contaminantions have been reported (Bakirdere and Yaman 2008) as being sources of pollution in both soil and ground waters; including exhaust emissions of vehicles, domestic and industrial wastewaters, and various solid wastes initiated from industrial production, fertilizers and pesticides. Industrial developments constitute significant sources of metals; which include mining, smelting and combustion of fossil. In addition, sources of heavy metals associated with automobiles

on roadsides, such as components wear, fluid leakage and corrosion of metals are other major metal pollutants of the roadside environments (Friedland, 1990; Wong *et al*., 2006; Akbar *et al*., 2006; Kummer *et al*., 2009 and Hua *et al*., 2009).

One of the promising approaches for heavy metals removal is "*phytoremediation*" (see Chapter 2 for more details), which is applied in this study. It is either to remove pollutants from the environment or to render them harmless (Salt et al., 1998; Salt *et al.,* 1995; Motesharezadeh et al., 2010). It is proposed to find ways to enhance the rate of metal removal from the contaminated soils, and hence, the selection of a suitable plant species is an important practice for successful application of phytoremediation techniques. For example, addition of green waste compost, chelating agent (EDTA), and/or bacteria may enhance heavy metals uptaking by plants (Wong, et al. 2006; McGrath and Zhao, 2003).

Phytoremediation has received increasing attention as a promising cost effective alternative to conventional engineering-based remediation methods (Salt *et al*., 1998). It is considered as an environmental friendly, aesthetically pleasing approach and socially accepted technology to remediate polluted soils (Ghosh and Singh 2005a; Garbisu et al. 2002). Chehregani, Noori and Yazdi (2009) used phytoremediation to find accumulator plants in a dried waste pool of a lead and zinc mine in Iran. It is concluded that "*N. mucronata* is an effective accumulator plant for phytoremediation of heavy-metals-polluted soils".

In Libya, vehicle emissions are one of the major pollution, which are the main reason for increasing the levels of lead elements in soils. This is due to the fact that

vehicles fuel is leaded. To the author's knowledge, no investigation has been carried out in this area. Therefore, this study aims to investigate the effects of heavy metals accumulation alongside the roads which was caused by vehicles' emissions and/or industrial activities in Libya. . The study puts forward a framework for *phytoremediation* approach as the basis for investigating the effects of heavy metals on the environment and on health. The next section will look at the characteristics of the study area that informed its selection for this study.

1.2. Study Area and its Environmental Problems

Azzawiyah city is located in the west of Tripoli (the capital) between 25° 00'N Latitude and 17° 00' E Longitude. The population of the city was estimated to be about 300,000 people in 2008 (Azzawiyah City Council Documentary 2010). The proposed area chosen for this study is about 4 km^2 mainly on the roadsides in the main Highway Road of Azzawiyah. This area is exposed to a wide range of pollutants derived from vehicular traffic. Consequently, there has been a governmental concern about the accumulation of the likely released heavy metals that may cause harmful side effects to both humans and other living organisms.

1.3 Aim

The aim of this study is to investigate soil pollution as result of industrial activities and vehicular traffic on agricultural land in Azzawiyah, Libya with the view of assessing potential application of phytoremediation option to remediate the

contaminated soils and determine whether soil amendments would improve soil remediation.

1.4 Objectives of Study

The main objectives of this study are as follows:

I. To characterise the chemical and physical properties of soils along the Azzawiyah road to form the basis of a case study.
II. To evaluate three plant species (Indian mustard (*Brassica Juncea*), *Eucalyptus camaldulensis*, *medicago sativum* for their potential use in remediation of heavy metal contaminated soils.
III. To examine the impact of chemical chelating agents and role of soil bacteria for metal availability in heavy metal contaminated soils and heavy metal uptake by plants.

1.5 Structure of the Thesis

This thesis comprises nine chapters. Chapter one provides a brief description of the location of the study area and the environmental problems associated with this area. It then moves on to present the aims and the objectives of the study. Chapter two is concerned with a review of the relevant literature concerning the soils, such as definitions and classifications of the heavy metals. This chapter also highlights the soil contamination, mobility of heavy metals and the onventional remediation technologies, where phytoremediation methods have been detailed. Finally, metal accumulating plants and effect of soil amendments on lead uptake are also presented.

Chapter three is divided into two sections. The first section presents the study context and the methods applied to investigate the study area. Section two summarises the results of the study area. Firstly, the chemical and physical properties of various types of soil are presented. Secondly, the heavy metals in soil samples are illustrated. Chapter four details the plant species used in this study for their “phytoremediation” of heavy metal contaminated soils. The chapter then moves on to describe the greenhouse-based experiments, which have been designed to investigate the uptake of heavy metal (Pb) by “hyperaccumulating” plants.

Chapter five reports the study of Pb uptaken from contaminated soils by various plant species under controlled factors soil conditioning. It also describes the greenhouse-based experiments designed to improve both chemical and physical properties of soil, as well as the metals uptake by accumulating plants. Then, the findings of the lead accumulated in the plant species are presented.

Chapter six reports the recovery of the lead accumulated during the “phytoremediation” process. Chapter seven presents a general discussion of the results for the conducted work as a whole where the obtained results of various experimental works were correlated with each other in order to draw generalised conclusions and findings. Finally, Chapter eight reviews the drawn conclusions and remarks of conducted study work.

Chapter 2

Literature Review

This chapter will provide an overview of theories concerning soil contamination with an emohasis on those caused by human activities. The chapter will begin by offering a brief distinguish between the two types of contaminations. Following this, heavy metal will be detailed incuding definition, clssifications and problems caused to the envirnonment. Next is a detailed description of soil contamination and related issues. The chapter then moves on to detail issues related to trace metal contamination of the urban soil environmentand and mobility of heavy metals followed by decribtions of the remediation methods for treating contaminants in soil and water. Following this, issues related to metal accumulating plants and plant species for phytoremediation, and factors influencing heavy metal availability and their uptake by plants will be highlighted. The chapter then offers a describtion of soil extraction methods. Finally, a discusson of soil amendments effect on lead uptaking and phytomining method will be provided.

2.1. General review of soil contamination

Most plants and animals depend on soil, as a growth substrate, for their sustained growth and development. In many instances, the sustenance of life in the soil matrix is adversely affected by the presence of deleterious substances or contaminants. These pollutants can be broadly classified into two groups: (1) organic; which contains carbon, and (2) inorganic, devoid of carbon (Webber and Singh, 1995).

The entry of contaminants into the environment results from either natural processes or human activity. Natural contamination originates from either excessive weathering of mineral and metal ions from rocks or from displacement of certain contaminants from the groundwater or subsurface layers of the soil. The most common human-assisted routes for entry of inorganic contaminants, and heavy metal in particular, into agricultural and non-agricultural lands are via transportation emission, mining activity, disposal of industrial effluents, sewage sludges, energy production and agricultural activity (McCutcheon and Schnoor, 2003).

The metal species commonly found in the soil as a result of the aforementioned human activities include copper (Cu), lead (Pb), zinc (Zn), nickel (Ni), cobalt (Co), and cadmium (Cd). Although some of these metals are required in trace amounts by living organisms for their normal physiological activities, excessive accumulation is toxic to most life forms. The problem of heavy metal toxicity is further aggravated by the persistence of the metals in the environment. For example, Pb can persist in the environment for 150-5000 years (Fridland, 1990). There is therefore, a pressing need to deal with the problem of excess metals already present in the soil and to prevent future contamination.

2.2. Heavy Metals

Alan (1995) defined the term “heavy metals”, which are considered as a part of inorganic pollution, as metals which have densities greater than a certain value (usually 5 or 6 g cm^{-3}). Chwalker *et al*. (2006) bring another definition of metal. They define it as an element that has a characteristic lustrous appearance and is a good conductor of electricity. This term

‘heavy metals’ is widely recognised, and used to describe all metals and metalloids associated with pollution and toxicity.

Heavy metal pollution is one of the major environmental problems in the world, and is present in every place in the modern industrial environment (Mohammad, 2008). Some of these metals are natural components of the soil and present mainly in the forms that are available for organisms (Ross, 1994). They are mainly introduced into the soil through either the disposal of liquid wastes, which leads to the flow of water bearing heavy metals from liquid waste to the ground, or local industrial processes and geological weathering. Automobiles are also major soil contaminants, in addition to gas consumption, various oils, and wear and tear, are all factors that contribute to soil pollution.

Metals are held within the soil by a number of mechanisms involving: (1) specific adsorption of metal cations on oxide and hydroxide surfaces, (2) non-specific adsorption of metal cations to the permanent charge sites of silicate clays and the pH, dependent on organic matter, (3) organic complication whereby humic acids absorb metals by the formation of chelate complexes, and (4) co-precipitation occurring when a chemical reagent is simultaneously precipitated in conjunction with other elements (Fine, et al. 2014; Pandey 2012).

The consequencies of such heavy metals pollution can cause risks for human, environment and other organisims (Sezgin *et al.*, 2008; Mohammad, 2008). These metals are mainly released and moved into the soil and concentrate in the food chain (Sezgin *et al*., 2008). This means that these metals can enter the body indirectly through food chain, and directly via the ingestion and inhalation of soil particulates (Harrison and Laxen, 1981). The

result can cause chronic or acute diseases (Sezgin *et al*., 2008). In addition, heavy metals may be leached from the soil in to water courses and may cause contamination to drinking water supplies,

2.2.1. Classification of Heavy Metals

Heavy metals, that are referred to in this study as contaminants of concern are, Cr, Cu, , Ni, Pb, Cd and Zn. These metals are known to be present in soil near to roads (Kummer *et al.*, 2009; Hua *et al*. 2009; Jaradat *et al*. 1998) and their presence poses a serious human health and environmental concern (e.g. lead and cadmium). Such a focus is because of their known toxicity with regard to human health or their ability to bio-accumulate and move through the food chain (Wong *et al*., 2005). These pollutants may come from automotive parts such as tread and brake linings that contain a variety of heavy metals, such as, Cd, Cr, Ni, Cu, Zn, and Pb. These heavy metals (e.g. Cd, Cr, Ni, Pb) emission from road transport, which can lead not only to environmental damage but also to a variety of adverse health effects (Kummer *et al.*, 2009).

Cadmium (Cd):

Cadmium is considered to be one of the most toxic metals (Paul Degobert, 1995). It is usually present as complex oxides, sulphides, and carbonates in Zn, Pb and Cu ores (Fergusson, 1990; Raymond, 2007). This metal is similar to zinc, in atomic structure and chemical behaviour (Berman, 1980). It is extremely toxic to most plants and animal species particularly in the form of free cadmium ions (Fergusson, 1990).

Cadmium is more mobile in aquatic environments than most other metals and is bio-accumulative and persistent. Cd is used in nickel-cadmium batteries, in metal plating, in pigments for glass, and as a stabiliser in polyvinyl chloride. Also cadmium can be emitted from zinc- based additives in lubricants, where cadmium is an impurity combined with the zinc. It is also emitted by wearing of vehicles tyres (Nabulo *et al*., 2006; Raymond, 2007).

The natural cadmium concentration has reported between 0.1-1.0 μg^{-1} and the average concentration in many countries in the world is 0.62 μg^{-1}. For example, the concentration of cadmium near to some roads in the USA ranges between 0.22 - 1.45µg/ g while cadmium concentration in England is in the average of 1.6µg/g (Fine, et al. 2014; Babu, Kim and Oh 2013).

In sediments, cadmium does not appear to be absorbed to colloidal material, however, organic matter (e.g. humic substances and organic debris) appear to be the main sorption material for the metal. The sorption of cadmium to sediments, and to the clay content, increases with increasing pH. The release of cadmium from the sediment is influenced by a number of factors including acidity, redox conditions and complexing agents in the water. Under alkaline conditions, cadmium is less mobile (Fergusson, 1990).

Chromium (Cr):

Chromium is one of the most abundant heavy metals in the lithosphere (Callender 2003), and is moderately toxic to aquatic organisms (Raymond, 2007). It is dominated by input from rivers, urban runoff, domestic and industrial wastewaters and sewage sludge (Denton et al. 1997). Waste stream from electroplating and metal finishing industry are the

other major sources of chromium in the aquatic environment (Callender 2003; Finkelman 2005; Klaassen, 2001).

Chromium is dangerous to humans and long term exposure has been associated with lung cancer in workers exposed to levels in air that in the order of 100 to 1,000 times higher than usually found in the environment (Finkelman 2005).

Copper (Cu):

Copper is one of the most important and essential trace elements for plants and animals. In nature, Cu forms sulphides, sulphates, sulphosalts, carbonates and other compounds (Alloway, 1995; Domyc, 2001). In nature, copper can be found in various sources including mining, smelting, domestic and industrial wastewaters, steam electrical production, incinerator emissions, and the dumping of sewage sludge (Denton et al. 1997). It is reported that the adsorption behavior of copper in natural systems is strongly rely on the type and concentration of inorganic and organic ligands (Finkelman 2005).

For various types of soils, it is reported that its concentration is in the range of 30 mg/kg, as it is associated with soil organic matter, oxides of iron and manganese oxides, soil silicate clays and other minerals (Alloway, 1995). Copper is essential for good health. However, exposure to higher doses can be fatal. Long term exposure to copper results in nose, mouth, eyes and irritation, and cause headaches, dizziness, nausea, and diarrhea (Finkelman 2005).

Lead (Pb):

Lead is one of the most toxic elements. It is widely naturally distributed; however, its greatest risks normally arise from emissions to the environment (Duffus, 1980). Organolead

compounds, on the other hand, particularly the alkyl-lead elements used as antiknock agents in gasoline, are considered toxic to any forms of life (Denton et al. 1997). The main sources of Pb in natural waters include manufacturing processes (especially metals), atmospheric deposition (e.g. from pyrometallurgical nonferrous metal production; the combustion of leaded fuels; the burning of wood and coal; and the incineration of municipal refuse). Other sources include domestic wastewaters, sewage and sewage sludge (Denton et al. 1997).

The average lead concentration in the soil surface is 10 - 20 ppm, and when the level arises to more than 100 ppm it gives indication of contamination (Fergusson, 1990). Lead is immobile in the aquatic environment and is still found in top soils that were previously contaminated (Fergusson 1990). Han *et al*. (2008) noted that Pb levels were the highest (e.g. 177ppm) at highway sites which have higher traffic density. This high concentration is attributed to motor-vehicle exhausts.

Lead enters the body through breathing or swallowing and the main target for lead toxicity is the nervous system. Lead exposure may also cause weakness in fingers, wrists, or ankles and miscarriage for pregnant women (Finkelman 2005). In addition, it is of particular concern because there is increasing evidence that relatively low concentrations of lead in the blood can affect children's mental development, an effect that persists into adulthood.

Nickel (Ni):

Nickel is moderately toxic to most species of aquatic plants, though it is one of the least toxic inorganic agents to invertebrates and fish (Fergusson 1990). Nickel pollution on a local scale is caused by motor-vehicles, such as corrosion of nickel from different vehicle parts (Finkelman 2005; Domyc, 2001).

The average concentration of Ni in world soils is probably around 20 mg/ kg (Alloway, 1995; Domyc, 2001), which obscures much variation between soil types. The content of Ni in a soil is considerably dependent on the nature of the parent material (Alloway, 1995). Major uses of nickel include its metallurgical use as an alloy, plating and electroplating, a major component of Ni-Cd batteries and the preparation of catalysts for hydrogenation of fats and methanation, and it is found in a wide variety of commodities such as automobiles, batteries and coins (Domyc, 2001).

Some of the most serious health effects of the exposure to nickel include reduced lung function. Moreover, some nickel compounds are reported to be carcinogenic to humans and metallic nickel, is also carcinogenic (Finkelman 2005).

Zinc (Zn):

Zinc is an essential trace element for humans, animals and higher plants (Alloway, 1995). Zinc is ubiquitous in the environment and is one of the components of tyres, which is released as they wear. Although Zn is an essential element for higher plants, it is phytotoxic at elevated concentrations, and consequently can reduce crop yields and soil fertility (Hua *et al*., 2009).

Most of the Zinc sources include the discharge of domestic wastewaters; coal-burning power plants; manufacturing processes involving metals; and atmospheric fallout (Denton et al. 2001). Approximately one third of all atmospheric zinc emissions are from natural sources, the rest come from nonferrous metals, burning of fossil fuels and municipal wastes (Denton et al. 2001). Furthermore, Zinc is a component of tyres, which is released as they wear (Nabulo, et al. 2006; Hua *et al*., 2009).

Zinc is an important component of huma and animal diet as it acts as a catalytic or structural component in numerous enzymes involved in energy metabolism (Alloway, 1995). However, taking excess zinc into the body through food, water and dietary supplements can have adverse effects on health. Ingesting high levels of zinc for several months may cause anemia, damage to pancreas, and decrease levels of high-density lipoprotein (HDL) cholesterol (Finkelman 2005).

2.3. Soil Contamination

The continued presence contaminants in soils stay much longer than any other compartments of the biosphere, whilst trace elements appear to be almost permanent in soil. Metals accumulated in soils can be depleted slowly by leaching or plant uptake (Kabata-Pendias and Pendias, 2001). Kabata-Pendias and Pendias (2001) also stated that the soil pollution or soil contamination occurs when an element or substance is present in soil greater than natural back ground concentrations.

2.3.1. Source of Metal Contamination within the Environment

The sources of heavy metal within the environment may be divided into two groups:

a) Natural or geological: Those released by the weathering and erosion of geological materials

b) Anthropogenic: Those released by human activities.

Anthropogenic sources of contamination can affect surface soil and vegetation. The most important sources of heavy metal are transportation, mining activity, energy production, and agricultural activity. A summarises of the main sources of metal contamination within the environment are shown in Table 2.1.

Table 2.1: A summary of sources of heavy metals in the environment (Martin *et al*., 1982)

Highway Contamination*:*

The highway contamination produces a number of heavy metal pollutants that arise from fuel combustion, wear and tyre abrasion. The extent of contamination is dependent on a number of factors including, average daily traffic density, wind direction, and topography (Martin et al., 1982; Abdelaziz *et al.* 1998 and Wong *et al*., 2006.

Energy Production and Mining Activities

Energy production: in coal-burning power stations fly ash, in a particulate form, is emitted and deposited into the surrounding environment. Coal fly ash contains a number of elements that include, Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Pb, and Zn that can potentially contaminate terrestrial systems

Surface mining can produce large quantities of waste and then leach to the environment. Mining activities including ore extraction, smelting and crushing can release high levels of metal in to environment (Martin *et al*., 1982).

2.3.2 Road Side Soil Contamination:

Road transport is one of the major sources of air pollution caused by emissions of vehicles. One of these pollutions is heavy metals that are initially not associated with traffic (Kummer *et al*., 2009). These heavy metals may cause adverse consequences and toxicity to both environmental quality and human health. Furthermore, Bakirdere *et al.* (2008) observed that cadmium levels in road side soils were generally decreased with distance from the main road. This decrease in the cadmium levels indicated that vehicular emission has a significant role in the levels of cadmium sustained in roadside soils. Akbar *et al*., (2006) showed that

heavy metals contamination in the soil represented in the road side verges in the study area was apparently higher as compared to the background levels for lead, cadmium, copper and zinc. “Lead concentration was the highest in the soil and ranged from 25.0 to 1198.0 µg/g (mean, 232.7 µg/g). Zinc concentration ranged from 56.7 to 480.0 µg/g (mean, 174.6 µg/g) and copper concentration ranged from 15.5 to 240.0 µg/g (mean, 87.3 µg/g). Cadmium concentration was the lowest in the soil and varied from 0.3 to 3.8 µg/g (mean, 1.4 µg/g)” (Khalid *et al*. 2006). This observation may be due to the higher input of these metals into road side environments by motor vehicles.

As stated earlier in section 2.2.1, heavy metals, that are referred to in this study, include Cd, Cr, Cu, Ni, Pb, and Zn. These metals are well-known to be existed in soil nearby roads, and their presence poses serious human health and environmental risks where lead and cadmium appear to be the most dengourse ones (Wong *et al*. 2005).

Sources of Road Side Pollutants

Recently, there has been concern with regard to the pollution caused by vehicles, which is due to the harmful metals emitted by vehicles. The majority of these metals are toxic to the living organisms, and even those are considered as essential can be toxic if they present in excess. The heavy metals can impair important biochemical processes posing a threat to human health, plant growth and animal life. These pollutants can be harmful to the roadside vegetation, wildlife, and neighbouring human settlements. The distribution of the heavy metals concentration in roadside soils is considerably high, but inversely correlated with the increase in the distance away from roads and traffic density (Akbar *et al*., 2006).

The sources of roadside contamination include:

1. Vehicle traffic,
2. Exhaust emissions,
3. Degradation of vehicle parts,
4. Atmospheric depositions,
5. Load losses from vehicles,
6. Losses from lubrication systems, and
7. Agricultural activities.

These sources can be summaries into four groups (Table 2.2), as follows.

1. Traffic from the operation and passage of vehicles including those arising from abrasion, corrosion and attrition both vehicles and highway surfaces,
2. Discharges from accidents spills,
3. Maintenance from operation carried out on road, and
4. From other miscellaneous sources; such as illegal disposal, maintenance of vehicles, and agricultural activities.

Table 2.2: The potential sources of metals within roadside environments (Luker *et al*., 1994).

Sources Generated from Internal Vehicular Pollution

Pollutant contributions caused by internal vehicle emissions initially originate from the metal content of fuel, engine components, and lubricating oils. These include

polyaromatic hydrocarbons (PAH), derived from incomplete combustion. Moreover, there are other emitted materials which can be loss from fuel; such as exhaust gases and vapour, petrol additives and hydrocarbon, as well as small quantities of bromide and nitrate may also be included in exhaust emissions (Cowgill *et al*., 1984).

Petroleum Additives

Road transport has been a main source for lead emissions. Lead pollution on a local scale by industrial emissions and on a larger scale by emissions from vehicles that use leaded gasoline. Lead comes from the compound which is called Tetra-ethyl lead, which was used in the late 90s as an additive in petrol to prevent the engine from premature detonation (knocking). All EU countries have banned lead as additives (*Directive 2003/17/EC*), which stated that the maximum content of lead allowed is 5 mgPb / l. Nevertheless, unleaded fuel does not mean that there is no lead in petrol; crude oil contains lead as an impurity (Kummer *et al*., 2009).

External Vehicular Pollution

The main processes by which vehicles spread heavy metals, such as Pb, Zn, Cu, Cd, and Ni to the environment, are combustion processes, wear of cars (e.g. tyres, brakes, engine, etc), leaking of oil, and corrosion. Lead is released in combustion of leaded petrol, zinc is derived from tyres dust, and copper is derived from brake abrasion and corrosion of radiators. Heavy metals are also released due to weathering of road surface asphalt and corrosion of crash barriers and road signs. In addition to corrosion of vehicles, fuel and lubrication and brake wear are also large sources of pollution (Kummer *et al*., 2009). Tyre wear releases lead,

zinc and hydrocarbon in a particulate form. Table 2.3 shows the element composition of rubber tyres.

Table 2.3: The element composition of tyres (Zereini and Skerstupp. 1997)

Zinc and cadmium are used in the vulcanisation of rubber (Zereini and Skerstupp, 1997). The corrosion of vehicles release heavy metals that include, Pb, Al, Mn, Cr, Cu and Zn. Zinc and lead are derived from rust particles and flakes of paint, also the brake lining and pad wear releases Pb, Cr, Cu, Ni and Zn in the roadside. In vehicles, loss oil lubrication is considered as a source of organic contaminants, as it contains metals and oil of about 8.5 mg/kg PAH in road dust (Cowgill *et al*., 1984).

Accidental Spillages:

Spillages can be a form of chemical contamination. It can range from small losses of fuel from vehicles to oil tankers. Sludge and slurries can contribute to suspended solids loading in Libya. There are a lot of vehicles, which can drop small quantities of sludges retained in their tanks, which contains high quantities of heavy metal.

Road Surface Degradation:

Road surface degradation is a source of chemical contamination alongside these roads. According to an earlier research conducted on the metal content of asphalt (Cowgill *et al*., 1984), it was reported that about 95% stone material and 5% bitumen binder by weight. The bitumen binder may contain many elements such as Ni, V, Ca, Mg and Fe. Stone materials can adsorb metals such as Pb, Cr, V and Zn onto their surfaces, via hydroxyl group interactions (Lindgren, 1996).

As reported by Charlesworth *et al*. (2003), Zn and Cu may be derived from the mechanical abrasion of vehicles. It was also found that Zn and Cd are related to tyre wear, and especially Zn is also associated with spills on road surfaces. “However, there is little evidence for any relationship between the distribution of Cd and Zn commensurate with their deposition together on road surfaces due to the wear of tyres.” (Charlesworth, et al. 2003). The heavy metal contamination on the roadside is dependent on a number of factors including (Ward, 1989; Fergusson, 1990; Charlesworth *et al*., 2003; Hua *et al*., 2009):

1- Distance from roadside edges.

2- Particle size.

3- Average daily traffic.

4- Wind direction and topography.

Distance from Roadside Edges:

One diagnostic method to identify the source of heavy metals in soil is to observe the change in concentration of these metals in conjunction with the distance from the source (Hua *et al*., 2009; Nabulo *et al*., 2006; Khalid *et al*., 2006; Alkhlaifat *et al*., 2007). Those researchers have explained that there is a relationship between levels of heavy metals near

roadsides and the traffic flow on these roads. In their studies, Hua *et al*. (2009), Jaradat *et al*. (1998) and Elgamail, (2000) demonstrated that the concentration of Pb, Zn and Cd were found higher in the samples taken from soils at the railroad edges, and also these values decreased as the distance increased from the railroad. The results of their studies are summarised in Table 2.4 (Hua *et al*., 2009).

Table 2.4: Heavy metal concentration in the railroad soils in mg/kg.

Country/Author	**Element concentration**	**Distance from roadside**	
		1.5 m	**10 m**
China Zhengzhou (Hua *et al*., 2009)	Cr	54.59	69.20
	Cu	44.09	42.80
	Ni	17.58	23.71
	Pb	146.80	138.87
	Zn	512.59	402.27
	Cd	0.45	0.38
Jordon (Jaradat *et al*., 1998)	Cu	44.6	23.2
	Cd	1.15	0.57
	Pb	272.2	28.8
	Zn	140.8	81.1
Egypt Cairo (Elgamail, 2000)	Cd	2.25	0.60
	Pb	875.8	180.90

Air Particle Size

The exhaust gases can migrate with airflow and deposit in roadside soils. The released gases from automobiles comprise of 40% particles are larger than 9 um in diameter, 20% of the particles are 1-9 um, and 40% of the particles are smaller than 1 um. The larger particles deposit mainly close to the road edge, while the smaller ones can stay suspended in the air for longer times and deposit at farer sites from the road. Particles of various sizes may deposit in areas within 50- 100 m away from the road (Hua *et al*., 2009).

Average Daily Traffic.

Spatially, the total concentration of heavy metals have been related to industrial and residential areas, as well as traffic movement, numbers of vehicles and their speed. A trend for higher concentrations of heavy metals was found on streets where traffic was more likely to undergo stop-start manoeuvres such as at traffic lights (Charlesworth *et al.*, 2003).

Abdelaziz *et al*. (1998) found that lead accumulation in urban environments is strongly related to the vehicular traffic density. Moreover, Abdelaziz *et al.* (1998) also found that Pb and Cd levels in vegetation increased with traffic density and proximity to roadways and then decreased as the distance increased from the roadside.

Wind Direction and Topography.

The dispersion and deposition of metal enriched particulates and dust in environment are governed by physical and micro-environmental factors; including topography, wind direction, and urban runoff. Wind direction in the environment is highly influenced by the positioning and topography of buildings. In the presence of buildings, air movements may

become channelled and/or confined at a limited street corner. All of this can cause changes in the wind speed and direction. This could subsequently affect the dispersion and deposition of dust and particulates, resulting in preferential deposition of heavy metals; where stagnant metal laden particulates concentrate (Wong *et al*., 2006). Moreover, dust and particulates on paved surface can be readily re-suspended by wind and can be easily swept by urban runoffs (Figure 2.3).

Figure 2.1: Processes and transport of metals in urban settings (Wong *et al*., 2006).

2.4 Trace Metal Contamination of the Urban Soil Environment

Soils serve as the most important sink for trace metal contaminants of the ecosystem, especially in the urban areas. The existence of these contaminations in the urban area

represents as a source of trace metals, which may have a long half-life of perhaps several hundreds of years (e.g, Pb). Urban soils are, therefore, an important indicator of human exposure to trace metals in the terrestrial environment. Irrespective of their functionality, they are highly susceptible to physical disturbance and chemical contamination due to their proximity to intense human activities. Unlike soils in rural and suburban areas, in the urban environment open or exposed soils, with or without vegetation, are usually fragmented and small in size because of urban planning, facilities, such as playgrounds and parks, where they are used as a substrate to grow plants for buffering and aesthetic purposes. Also, chemical contaminants can be found in private backyards or in small plots used to grow food (Wong *et al*., 2006).

Heavy metals in urban area are considered to be one of the main sources of environmental pollution (Alkhashman *et al*., 2005; Aydin *et al*., 2007; Nabulo *et al*., 2006). These pollutants can also bio accumulate in plants (Oliva *et al*., 2007). In urban agriculture, it is also considered a potential pathway for trace metals to transfer from motor vehicle emissions to soil and food crops grown along road verges (Nabulo *et al*., 2006). Atmospheric pollution is one of the major sources of heavy metal contamination in soils and roadside dusts in urban areas. Soil in an urban environment may have a direct influence on human health, which it is important to assess the possible sources of pollutants in urban soils (Oliva *et al*., 2007).

Some studies have shown that urban soil can receive large inputs of trace metals from different anthropogenic sources but especially from automobile emissions. Plant growing in contaminated environments can accumulate trace elements at high concentrations, causing a serious health risk to consumers (Nabulo *et al*., 2006). The main sources of street dust, and

consequently of the trace elements found therein, are deposition of previously suspended particles (atmospheric aerosol) and supplanted urban soil. Additionally, the emissions from several point sources (vehicular traffic, heating system, building deterioration, construction and renovation, corrosion of galvanised metal structures (Baptista *et al*., 2005).

Elevated levels of trace metal contents are ubiquitous in urban settings, this has aggravated as a result of the wide range of human activities in the urban settings including industrial, municipal, residential, and traffic related uses (Alkhashman *et al*., 2005; Aydin *et al*. 2007). As a consequence, the adverse effects of poor environments on human health are most evident in urban environments. Excessive emissions of trace metals, often in the form of particulates, contaminate the environment surface including air and deposits of the land (Aydin *et al*., 2007). The composition and quality of urban road dusts are indicators for environment pollution, interest in the levels of contaminants associated with urban road dusts, particularly Pb, has risen in the last few decades. Reduced cognitive development and impaired intellectual performance of children have been linked to Pb exposure. Other elements, such as Cu, Cd and Zn are also well-known toxic elements (Han *et al*., 2008).

Interest in the levels of pollutants associated with the dust of the street has increased in recent decades, especially in light of the impact of high blood lead levels in children living in urban areas (i.e. this is possibly due to unintended consequence of the hand and mouth, while children play in a city street). Many studies throughout the world have identified the sources of these contaminations in street dust as those associated with vehicular traffic, industrial and residential areas, as well as weathering of building facades. These studies have, in fact, recognised street dust as a significant pollution source itself (Charlesworth *et al*., 2003; Alkhashman *et al*., 2005; Nabulo *et al*., 2006).

In particular, high traffic volumes in urban areas are responsible for increased particulate contents within a breathing zone, because the turbulence in the near-surface atmosphere and suspension of particles from the road surface. The region within 150m from high way is at the highest risk of contamination with re-suspended particles from roadside sediments. If residents live close to busy roads, then they are exposed to high levels of metals, this can have a negative effect on their health. Environmental and health effects of trace metals in roadside sediments depend on their mobility and bio-availability of the elements, which are a function of their partitioning within sediments (Lee *et al*., 2005).

Metals are non biodegradable and accumulative in nature elevated emissions, their deposition overtime can lead to anomalous enrichment, causing metals contamination of the surface environment. Figure 2.2 shows the major compartments of an urban environment (Coby *et al*., 2006).

Figure 2.2 . The major compartments of urban environment (Coby *et al*., 2006).

2.5. Mobility of Heavy Metals

The mobility of heavy metals in soil is one of the key properties that determine their concentrations and their structure in a soil profile. They become readily available to plants; this causes heavy metals to enter the food chain and they can also migrate to groundwater. Specific soil properties, mainly its pH, Eh, cation exchange capacity, amount of organic matter in soil, amount of clay minerals and amount of iron, manganese, and aluminium oxides, control the rates of heavy metal migration into soil profiles. Clay minerals, Fe, Mn, Al oxides and organic matter are the most important groups for the sorption of heavy metals (Wong *et al*., 2005).

In general, metals added to soil initially settle at the soil surface. Their movement to groundwater, surface water, or the atmosphere is minimal as long as the retention capacity of the soil is not exceeded (Wong *et al*., 2005).

Metals movement in soil is directly related to the surface chemistry of the soil matrix and soil solution. The concentration of metals in the soil solution, at any given time, is governed by a number of interrelated processes, including inorganic and organic complexation, oxidation-reduction reactions, precipitation/dissolution reactions, adsorption/desorption reactions, pH and redox potential of the soil waste matrix (McLean and Bledsoe, 1992).

Brown *et al.* (1997) studied the effect of alkalinity on the metal mobility; the obtained result showed that the mobility of most metals was enhanced with increase of pH. The bio-solids (lime-stabilised) was amended soil site. The lime added may neutralise subsoil acidity to 70 cm below the soil surface. The pH in soil was as low as 5 between 40 to 50cm, while

the pH was higher in soil layer lies between 80 to 90cm. All three metals (Cu, Pb, and Zn) were detected in soils of high pH values. The concentration of these metals was determined at different depths and then compared to control values. The concentration of these metals at different depths was found to be high compared to the control value. This confirmed the mobility of these metals. The authors explained that the movement of these metals caused the formation of fulvic acids and metal complexes. Moreover, when the soil pH increases then the solubility of fulvic acids will increase.

Fine, et al. (2014) reported that the mobility of most heavy metals in the soil and subsoil profiles depends on the physical and chemical properties of the metal in both liquid and solid phases. Many chemical changes may occur during the movement of water through the soil including dissolution or precipitation, adsorption/ desorption, degradation, filtration and a variety of transport processes which indicate significant differences in mobility among heavy metals.

In a similar study, Kumar, et al (2013) concluded that the association of metal ions with the mobile colloids increased the mobility of these metals through the soil. They also found that pH and ionic strength are important factors which affect the mobility of metals in soil. Moreover, the authors proposed several mechanisms to enhance the mobility of metals in porous media, which are associated with inorganic, or with colloids, and might provide a faster transport mechanism.

Amrhein *et al.*, (1993) stated that the enhancement of metal leachate from the soil has been associated with presence of colloidal particles, which are made up of organic matter and iron oxides. It was also noted that the mobilisation of colloid sized particles occurred under

low salinity; this means that the mobility of these trace metals and colloidal particles increased when the salinity decreased. Mobile colloids may aid in transporting heavy metals in soils, however, the mobility of natural colloids was proven by the presence of humic substances in deep aquifer. Humics were also found as coatings on accumulated clays and organic matter in the subsoil horizon of many soils. Colloids travel with water through soils (Ali, Khan and Sajad 2013). Similarly, Pandey (2012) discussed the percolation of the soil solution as a mechanism for the transport of the soil organic matter through soil. They concluded that this organic matter may form complexes with metal ions making them more mobile. It was also reported in this study that there was a good correlation between the concentrations of Pb, Cu, Zn and Mn with the concentration of soluble organic compounds, thus allowing the formed complex ions to have low molecular weight compounds.

2.6 Remediation

Remediation refers to processes or methods for treating contaminants in soil or water; so that the contaminants being removed and become less harmful. Soil remediation generally refers to processes that directly treat the medium and affect the contaminant in some way and it is divided in two categories; *In Situ* remediation and *Ex Situ* remediation as shows in Figure 2.3.

2.6.1 Conventional Remediation Technologies

Conventional technologies available for water and soil remediation can be broadly classified based on whether they are employed *in situ* or *ex situ*. *In situ* remediation refers to the treatment of soil in its original place, whereas, *ex situ* remediation involves physical removal and treatment of soil either on site or transported to another location (Gary *et al.*,

2005). *In situ* techniques are favoured over the *ex situ* techniques due to their low cost and reduced impact on the ecosystem (Ghosh and Singh 2005a; McCutcheon and Schnoor, 2003).

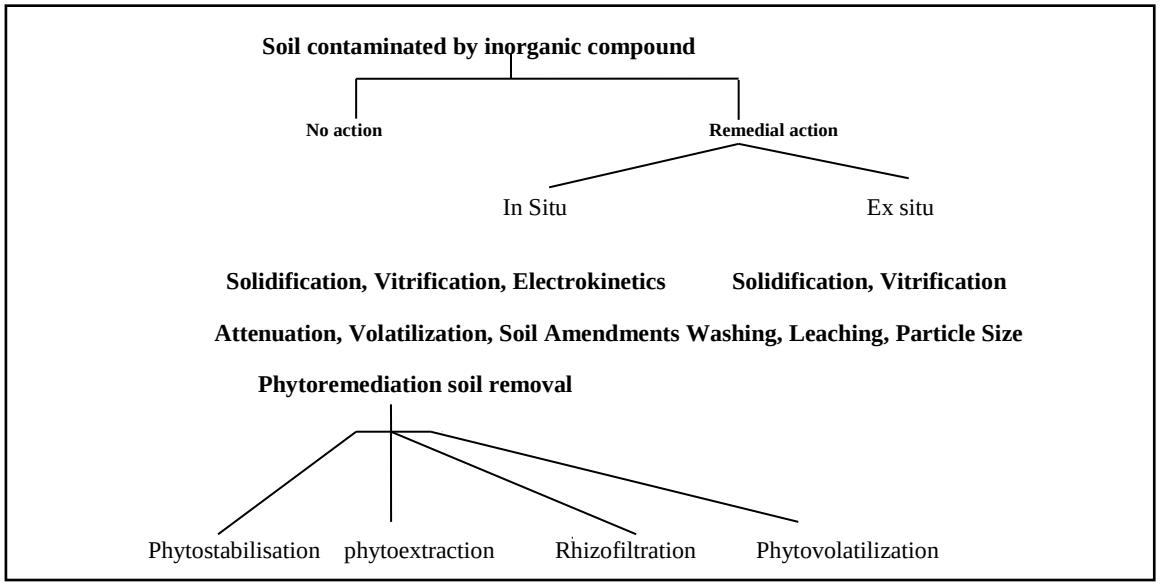

Figure 2.3: Classification of Remidiation Processes.

Soil Flushing

The process involves physical separation by vertical or horizontal leaching using a fluid (e.g. water or an aqueous solution containing chelators), followed by collection and treatment of the leachates in basins or trench infiltration systems.

Pneumatic Fracturing

The process involves injecting pressurised air into the soil to develop cracks in low permeability areas, thereby enhancing the extraction efficiencies of other *in situ* technologies (Ghosh and Singh 2005a).

Solidification/Stabilization

In these processes the contaminant is physically enclosed in a stabilised mass or through chemical interactions induced between the stabilising agent and the contaminant.

Vitrification

This technology utilises thermal energy to melt the soil to enable physical or chemical stabilisation (Ghosh and Singh 2005a).

Electrokinetics

The contaminants are mobilised as charged species towards polarised electrodes placed in the soil. The migrated contaminants can be removed or treated in situ (Ghosh and Singh 2005a).

Chemical Reduction/oxidation

In this remediation process, the contaminants are chemically converted into less hazardous, more stable, less mobile and/or inert forms (Ghosh and Singh 2005a).

Soil Washing

This process refers to the separation of contaminants adsorbed to fine soil particles using an aqueous solution, through size separation, gravity separation, or attrition scrubbing.

Excavation, Retrieval and Offsite Disposal

This method requires removal and transportation of the contaminated soil to an offsite treatment and disposal-facility. In general, all of these conventional technologies, which are colloquially kown as, "*pump-and-treat*" and "*dig-and-dump*" techniques, are limited in their applicability to small areas and have their own inherent limitations. In locations where the contaminants concentrations are slightly higher than the industrial criteria (i.e. governments regulations), use of conventional technologies is not economically viable. So far, irrespective

of the technology being selected, the cost estimates for utilising conventional remediation techniques have remained high. The overall remediation budgets includes design, construction, operation and maintenance costs of the process associated with each technology, in addition to mobilisation, demobilisation and pre and post- treatment costs which are determined on a site-to-site basis. Also, in the case of most *ex situ* treatment technologies, excavation and transport costs need to be factored in, to reach a final cost for remediating a contaminated site (Ghosh and Singh 2005a).

2.6.2 Phytoremediation

Phytoremediation consists of the Greek prefix phyto (plant) attached to the Latin root remedium (Cunningham *et al*., 1996). Phytoremediation is a preferable soil remedial technique to remove trace metals. It is also defined as the use of plants, sometimes in conjunction with microorganisms and chemical reagents, to clean up contaminated sites. With just a few years pytoremediation has bloomed into a number of interesting and potential applications of treating element contaminants from soil. Its emerging low-cost and ecologically friendly alternative to the conventional remediation technologies has gained a great deal of interest in both public and private sectors. Phytoremediation can be defined as the process of utilising plants to absorb, accumulate, detoxify and/or render harmless, contaminants in the growth substrate (soil, water and air); through physical, chemical or biological processes (Fine, et al. 2014; Ali, Khan and Sajad 2013; Pandey 2012; McCutcheon and Schnoor, 2003). The use of plants for remediating heavy metal contaminated soils has multifold advantages as follows:

1. large scale application, as plants can be sown or planted in large areas,
2. growing plants is relatively inexpensive,

3. plants provide an aesthetic value to the landscape of contaminated sites,
4. phytoremediation process is environmentaly friendly and ecologically safe,
5. some plant species, used for phytoremediation, can have potential economic returns which would offset the cost of the technology,
6. plants concentrate the contaminants within their tissues, thereby reducing the amount of hazardous waste, and
7. concentrated hazardous waste require smaller reclamation facilities for extracting the heavy metals (Ali, Khan and Sajad 2013; Pandey 2012; McCutcheon and Schnoor, 2003).

Apart from the direct advantages, plants provide indirect benefits to the contaminated sites such as:

1. increased aeration of the soil, which in turn enables microbial degradation of organic contaminants and microbe-assisted uptake of metal contaminants,
2. reduced top soil erosion due to plant stand,
3. enhancement of rhizospheric micro-fauna and flora for maintaining a healthy ecosystem (Fine, et al. 2014; McCutcheonand Schnoor, 2003).

In addition, phytoremediation can be readily applied to restore contaminated soil at any site. Hyperacumulators are used as well as other high biomass plants (Wong *et al*., 2006). It is necessary to use phytoremedation to allow the renewed soil to have its original properties. The problem of concern in soil remediation actions is the cost. Phytoremediation methods are likely to be less costly than those based on conventional technology (Cunningham *et al*., 1996).

Phytoremediation approaches

Although the basic concept of utilising plants to remediate contaminated sites remains the same, phytoremediation technology can be subdivided into different approaches based on their underlying process and applicability, as follows:

Phytostabilisation

Involves the use of plants especially roots and/or plant exudates to stabilise, demobilise and bind the contaminants in the soil matrix, thereby reducing their bio-availability (McCutcheon and Schnoor, 2003). This approach is suitable for both organic and metal contaminated soils (King *et al*., 2008).

Phytovolatilisation

Phytovolatilisation involves the use of plants to take up contaminants from the soil transforming them to be volatile and transpiring them into the atmosphere. Plants extract volatile pollutants (selenium, mercury) from soil and volatilise them from the foliage. Some of these contaminants can pass through the plants to the leaves and volatilize into the atmosphere (Ghosh and Singh 2005a; McCutcheon and Schnoor, 2003).

Rhizofiltration

Rhizofiltration utilises plant roots to take up and sequester metal contaminants and/or excess nutrients from aqueous growth substrates (wastewater streams, nutrient-recycling systems) (McCutcheon and Schnoor, 2003). This approach is suitable for remediating most metals, i.e. Pb, Cd, Ni, Cu, Cr, V (Zacchini *et al*., 2009).

Phytoextraction

Phytoextraction involves specific plant species which can absorb and hyperaccumulate metal contaminants and/or excess nutrients in harvestable root and shoot tissue, from the growth substrate (soil) (McCutcheon and Schnoor, 2003). It uses plants to remove metals or organics from soil by concentrating them in the harvestable parts. This approach is suitable for removing most metals (e.g. Pb, Cd, Ni, Cu, Cr, V) from contaminated soils (Ghosh and Singh 2005a; McCutcheon and Schnoor, 2003; Brown *et al*., 1994; Unnisa *et al*., 2008).

Phytoremediation comprises four main processes, shown in Table 2.5. This study has focused on applying phytoremediation, especially phytoextraction, for remediating heavy metal contaminated soils.

Table 2.5: The main processes of phytoremediation (Ghosh and Singh 2005a*)*

2.6.3. Pytoextraction

Phytoextraction seems to be a simple and economic technique for the remediation of metal polluted soils (Nevel *et al*., 2007). Marchiol *et al*. (2007) also stated that phytoextraction refers to the use of pollutant accumulating plants extracting and translocating

contaminants to the harvestable parts. Phytoextraction involves the uptake of heavy metals and their accumulation in harvestable portions of plants to promote long term soil cleaning. The authors also stated that several plants can extract high amounts of metal from contaminated soil, which is called hyperaccumulating (Schmidt *et al*., 2003). Phytoextaction has been proposed as an inexpensive sustainable, *in situ* plant based technology, which makes use of natural hyperaccumulotors, as well as high biomass producing crops, to help rehabilitate soils contaminated with heavy metals without destructive effect on soil properties. Phytoextraction has received increasing attention as a promising cost effective alternative to conventional engineering-based remediation methods (Salt *et al*., 1998).

The phytoextraction process involves the use of plants to facilitate the removal of metal contaminants from a soil matrix (Kumar *et al*., 1995). If metal availability in the soil is not adequate for sufficient plant uptake, chelates or acidifying agents may be used to liberate them in to soil solution (Lasat *et al*., 1998). Phytoextaction should be viewed as a long term remediation effort requiring many cropping cycles to reduce metal concentrations. This technology is suitable for the remediation of large areas of land that are contaminated at shallow depths with low or moderate levels of metal contaminants (Ali, Khan and Sajad 2013; Rajkumar, et al. 2012; Kumar *at al*., 1995).

The success of phytoextraction is strongly determined by the amount of biomass and the bio-availablity fraction of heavy metal in the rooting medium and the concentration of heavy metals in plant tissues (Saifullah *et al*., 2009). Soils with a high degree of metal pollution can be revegetated by metal resistant plants, but their decontamination capacity is restricted by their low biomass production, so that decontamination of the soil cannot be achieved at a reasonable time. However, the revegetation of these soils avoids further

dispersal of metals by water or wind erosion (phytostabilisation). Consequently, the *in situ* application of such chelators could pose as an environmental risk of water pollution by uncontrolled metal solubilisation and leaching (Ali, Khan and Sajad 2013; Rajkumar, et al. 2012; Kayser *et al*., 2001).

The efficiency of phytoextraction is dependent on the metal concentration in shoots and high biomass production. In the case of low metal availability, the synthetic chelators (chelate phytoextraction), such as EDTA and nitrilotriacetic acid (NTA) are used and have been shown to increase accumulation of metal, such as Pb. The idea of using plants to remediate metal polluted soils came from the discovery of hyperaccumulators or other plant spices which is defined as plants often "endemic" to naturally mineralised soils which accumulate high concentrations of metals in their foliage (Ali, Khan and Sajad 2013; Rajkumar, et al. 2012; Pandey 2012; Kumar *et al*., 1995).

To overcome the limitations due to plant characteristics, different strategies have been suggested to improve the phytoextraction process. Brown *et al*. (1995) proposed to transfer the metal-removal properties of hyperaccumulator plants to high-biomass producing species. However, this approach is limited by the lack of information on the genetics of metal hyperaccumulation in plants, particularly the heredity of relevant plant mechanisms; such as metal transport and storage (Ali, Khan and Sajad 2013; Pandey 2012; Lasat *et al*., 2000).

2.7. Metal Accumulating Plants

Tree species generally absorb more water and minerals than annual crops, acting as efficient biological sieves to prevent wastewater from recharging into underground water reservoirs. Therefore, trees minimise mineral accumulation into the soil and protect ground

water from becoming polluted (Nelson, 1995). Biochemical changes occurring in the soils of tree plantations favourably contribute to nutrients mobility making them available to the plant and enhancing uptake, causing no detrimental effects on the site and the surroundings (Chabra, 1989). Moreover, they are good remediators as they are able to remove the heavy metals from polluted sites; providing an eco-friendly substitute for traditional removal heavy metals from the area (Dalun, *et al*., 2009).

Plant, which is typically considered good for phytoremediation, should be highly tolerant to the pollutant, able to accumulate high levels of pollutants in the biomass, have a scattered root system that is potentially able to uptake excessive quantities of water from the root zone, and fast growing with high potential for biomass production (Isaaa, 2006). It is interesting to note that many species are habitually tolerant and do well on polluted soils but physiologically, they are slow growing with low potential for biomass production, in fact, they become well adapted to a particular extreme environment. Conversely, a high biomass producing tree species having widespread roots, demanding nominal inputs for its establishment, has poor tolerance to contaminants, and cannot concentrate the pollutants. Therefore, traditional plants are not always successful as phytoremediators (Isaaa, 2006).

Metal-accumulating plants generally grow slowly, are smaller in size, and/or weedy in nature. Such plants potentially generate little biomass and their growth behaviour and requirements are indeterminate (Shah *et al*., 2008). Willow acts as biological filters for wastewater and sludge disposal and can grow on nutrient poor industrially-contaminated soils. These unique features can be used for Phytoremediation (Punshon and Dickinson, 1997). Disposal and utilisation of wastewater through tree plantations are managed by adjusting the total discharge of municipal wastewater in such a way that whatever the volume

of wastewater is received at the site must be utilised within 12 to 18 hours through evapo-transpiration leaving no trench or furrow filled with standing water. Consequently, there would be no breeding sites for mosquitoes and no foul smell in the surroundings. Such plantations will also result in effective recharge of the ground water table (Paramathma *et al*., 2003).

Some plants accumulate much higher concentrations of toxic elements in their above ground parts as compared to underground ones. Such plants are said to be hyper accumulators. Hyper-accumulator plants have the ability to take up contaminants / pollutants that are found in abundance in the soil medium. Roots, shoots and/or leaves are the possible organs for the accumulation of contaminants in the plant body (Baker *et al*., 1994; Raskin *et al*., 1994; Cunningham *et al*., 1996). Proportionate levels of Cd, Co, Cr or Cu, Ni, and Pb in dry matter of hyper-accumulator plants is reported as ≥ 1000 mg g-1 or ≥ 0.1% of dry matter; and of Mn, Zn is ≥ 1% or ≥ 10,000 mg g-1 of dry matter. The level of Cd is reported as > 0.01% by dry weight (Baker and Brooks, 1989).

2.8. Plant Species for Phytoremediation

The potential for any plant species to successfully remediate heavy metal contaminated sites depends on all of the following prerequisite factors: (1) the amount of metals that can be accumulated by the candidate plant, (2) the growth rate of the plant in question, and (3) the planting density. The growth rate of a plant in a contaminated soil is important from the perspective of biomass (Ali, Khan and Sajad 2013; Pandey 2012; McCutcheon and Schnoor, 2003).

In the natural setting, certain plants have been identified to have the potential to uptake heavy metals from the soil. About 45 families have been identified to

hyperaccumulate heavy metal some of them are *Brassicaceae*, *Fabaceae*, *Asteraceae scrophulariacea*e and *Lamiaceae*. *Brassica juncea*, commonly identified as Indian mustard has been indicated to have a good ability to transport lead from the roots to the shoots (Dushenkov, 2003). It is also described as an appropriate plant for phytoremediation (Terry *et al*., 1992). Plants used for extraction of metals from soil must be tolerant to the heavy metals, adapted to the local soil and climate characteristics, and able to take up a large amount of heavy metal (Keller *et al*., 2003). The use of hyperaccumulators for phytoextraction relies on their ability to absorb metal contaminants from the soil and to translocate them to aerial plant parts. The idea of using plants to remediate metal polluted soils came from the discovery of hyperaccumulators or other plant spices. The following three plants will be used for phytoextraction in this study.

2.8.1. *Indian Mustard (Bjuncea*)

In the family *brassicaceae* , also known as green mustard cabbage, which is grown as a green vegetable and for the production of oilseed. The *Brassicaceae* include high biomass crops that have a large biomass production but they take up lower metal concentrations than hyperaccumulators, and have been singled out for their potential in this regard (Kumar *et al*., 1995). Indian mustard was identified as a species that is able to take up and accumulate metal into above ground parts metals including Cu, Cd, Zn, Ni and Pb (Hagg-kerwer *et al*., 1999). A number of lists of promising plants for phytoextraction of metal are summarised in table 2.6.

2.8.2. Medicago sativa (alfalfa)

This plant may be a good source of plant tissues, because it has been found to tolerate heavy metal and grow well in contaminated soil (El-Kherbawy *et al*., 1989; Baligar *et al*.,

1993). In a similar study, Gardea-Torresdey *et al*. (1996) also have shown that alfalfa is a potential source of biomaterials for the removal and recovery of heavy metal ions.

Table 2.6: Promising plant for phytoextraction (Kumar *et al*., 1995 and Lasat *et al*., 1998)

2.8.3. *Tree Species*

In the recent years, fast growing trees species have also been suggested to be used for phytoremedation of soil contaminated by heavy metal (Almeida *et al*., 2007). Among these different types of trees species is *Eucalyptus*, which has been used in this research. Researches, as scholars like (Shukla *et al*., 2010), have recommended that the *Eucalyptus* is one of the appropriate plant species for phytoremediation because there are lots of benefits attributes with phytoremedation which include: high biomass production, high growth rate, a deep root system, high capacity to grow in soils with low nutrient availability.

In England, *Eucalyptus camaldulensis* is commonly named as "River red gum" and "Red gum". *Eucalyptus* may grow in various climatic conditions, from warm to hot and from sub-humid to semi-arid and both of permanent and seasonal climates. These plants are

distributed throughout Northern Australia and widely introduced to most countries in Southeast Asia and Africa. *Eucalyptus camaldulensis* commonly grows up to 20m in height, occasionally reaching to 50 m., with a trunk diameter of 1 m. In open formations have a short, thick bole and a large, spreading crown. For plantations, *Eucalyptus* has a clear bole of 20m with an erect, lightly branched crown; bark smooth, white, grey, yellow-green, grey-green or pinkish grey (Shukla *et al*., 2010). There has been an increase in the utilisation of fast enormous trees particularly *Eucalyptus*. This is as a result of its great strength and good durability. *Eucalyptus* is used as the raw material for the wood chip and pulp paper industry. The wood is suitable for many structural applications such as rail way, sleepers, poles and floorings wharves, ship building and heavy construction. It is also in high demand because of its uses as fuel wood, such as charcoal (Shukla *et al*., 2010).

2.9. Factors Influencing Heavy Metal Availability and their Uptake by Plants

The composition of the soil at a contaminated site can be extremely diverse and the heavy metals present can exist as components of several different fractions (Salt *et al*., 1995). Plants grown in metal-enriched substrates take up metal ions to varying degrees. This uptake is largely influenced by the bio-availability of the metals which is, in turn, determined by both external (soil-associated) and internal (plant associated) factor (Salt *et al*., 1995). The success of any phytoremediation scheme relies on the availability of metals from the soil, which in turn is controlled by, chemical (pH, Eh, CEC, metal speciation), physical (size, texture, clay content, % of organic matter) and biological (bacteria, fungi) processes and their interactions (Ernst, 1996). Water deficit and salinity are a major biotic stresses that limit plants productivity in many parts of the world, particularly and semi-arid regions. Whilst water deficit reduces water availability due the decreasing the osmotic potential of the rooting

zone solution, salt stress reduce osmotic effect or ion specific effect via extreme accumulation of ions in to the plant tissues (Munnus and Tester, 2008).

2.10 Soil Extraction Methods

The technique is widely used for soils to employ a single extractant whose content for one element correlates with the plants available content, and can be used to predict plants uptake or toxicity symptoms occurring in plants or animals (Alloway, 1995). It also indicates that the soluble or extractable metals are better indicators of the availability of metals for plants uptake (Alloway, 1995).

Bio-availability is the proportion of total metals available for incorporation in to bioaccumulation. Total metal concentration does not necessarily match with metal bioavailability. Total metal content is not a good indicator of exposure or risk (McLaughlin *et al*., 2000). The trace element availability has been used to describe the extracted amounts of trace element from the soil by a chemical extraction, and the results of those extractions are potentially available to plants. The bioavailability has been used to compare trace elements extracted from those materials by some living organisms, such as plants and biota.

Phytoavailability is specifically related to the extraction of those elements by plants (Alloway, 1995). The extraction methods outlined above have been widely and successfully applied in the study of nutrient element deficiency in agricultural crops and animals, and to some extent, in the assessment of potential toxicity from the element Ni occurring in natural concentration. For toxic elements such as Pb or Hg in soil, concentration is elevated by pollution from atmospheric sources and industrial effluents. (Ure, 1996; Pulford and Watson 2003; Keller *et al.*, 2003; Rosselli *et al*. 2003).

Several methods have been used to evaluate bioavailability of trace element in soils which are mainly based on extractions by various solutions such as acids at different concentration, chelating agents e.g., EDTA, DTPA, and neutral salts such as $CaCl_2$ (Gupta *et al*., 2007; Gupta and Aten,1993; Sahuquillo *et al*., 2003).

The increasing performance of the analytical techniques used for element determination in an extract, together with the increasing evidence that exchangeable metals better correlate with plant uptake, has led extraction methods towards the use of less and less aggressive solutions. Neutral salts dissolve mainly the caution exchangeable and the complexing ability of the anion which can play a certain role. The most common single extractions are: acid extractions such as (HCL, HNO_3), chelating agents such as (EDTA), buffered salt solution such as (NH4- acetate acetic acid) and unbuffered salt solution such as $CaCl_2$. Etter *et al*. (2007) stated that unbuffered salt solutions are widely used for the extraction of exchangeable methods; they also added that single extractions are a suitable method for extracting metals from the soil. It is concluded that the most commonly used mild extractants are $CaCl_2$ and $NaNO_3$ procedure which would be more protective because of its higher leaching capacity (Sahuqullo *et al* 2003).

In their study, Pueyo *et al* (2003) used three methods: 0.01 mol^{-1} $CaCl_2$, 0.1 mol^{-1} $NaNO_3$ and 1 mol^{-1} NH_4NO_3 to assess the metals and to extract Cd, Cu, Pb and Zn from the contaminated soil. They calculated the 0.01 mol l^{-1} $CaCl_2$ extraction procedures, which seem to be the most suitable method for performing a harmonisation process and a suitable method for the determination of Cd, Cu, Pb and Zn mobility in soil. It is apparent that a wide range of single extraction methods can provide the evaluation potential availability of soil pollutants for plant uptake. The dependable extraction could provide valuable information for predicting

metal availability to plants and extracts such as 0.01 M $CaCl_2$ for Cd, Cr, Cu and 1M NH_4NO_3 for Cd, Cu, Pb and Zn have been identified as suitable extracts for mineral soil (Hammer *et al*., 2002).

In this study, determination of the speciation of Pb, Cd, As, Cu, Zn and Ni was conducted following a single selective extraction, which has been used extensively in the study of soil chemistry and is applied to give an assessment of bioavailability. The single selective extractions ($CaCl_2$) were carried out in order to estimate the degree of mobility and bioavailability of heavy metal in soil samples collected from Azzawiyah area in Libya.

2.11 Effect of Soil Amendments on Lead Uptake

Heavy metal-contaminated soil is one of the widespread global problems. Removal of these persistent pollutants is necessary but is very difficult. The remediation of large volumes of such soil by conventional physicochemical technologies previously developed for small, heavily contaminated sites would be very expensive. Phytoremediation of heavy metal-contaminated soil is an emerging technology that aims to extract or inactivate metals in soils. It has attracted attention in recent years for the low cost of implementation and environmental beneficial. Moreover, the technology is likely to be more acceptable to the public than other traditional methods (Tandy *et al*., 2006). On one hand, as an important mechanism, phytostabilisation can reduce ecological risk of air and water pollution of heavy metals (Wel *et al*., 2006).

On the other hand, phytoextraction, mainly using hyperaccumulator to remove heavy metal from polluted site, is more important approach of cleaning contaminated soil (Wel *et al*., 2006). Apparently, phytoextraction is the most important way of phytoremediation to

remediate polluted soil. The hyperaccumulator means a plant that can accumulate extremely high quantities of metals in its above ground biomass and its key characteristics include critical concentration property, translocation property, tolerance property and accumulation coefficient property (Wel and Zhou, 2006). Although there are increasing reports on discovery of hyperaccumulators (e.g. Thlaspi caerulescens J. *et al*; C. Presl; Pteris vittata L.; Sedum alfredii H.) phytoextraction technology has not widely been used in remediation practice (Srivastava *et al*., 2009). The main limiting factor is the low remediation efficiency of hyperaccumulator due to limited accumulation concentration in its shoot and biomass. Some researchers were dedicated to explore the mechanisms of hyperaccumulation and subsequently to improve phytoextraction efficiency by trans-gene or beneficial microorganism. Unfortunately, the progress has been very slow (Doty, 2008). Thus, many studies have been focused on addition of natural and/or synthetic chelators to increase uptake and translocation of heavy metals from soil in order to achieve high removal rates. Several chelating agents, such as citric acid, EDTA, CDTA, DTPA, EGTA, EDDHA, and NTA have been studied for their ability to mobilise metals and increase metal accumulation in different remediative plants (Babu Kim and Oh 2013; Munn, 2008; Etter *et al*. 2007; Sahuqullo *et al* 2003).

In their study, Huang *et al*. (1997) investigated the effects of applying EDTA to a Pb-contaminated soil on Pb accumulation in bromegrass. The authors found that adding EDTA has increased shoot Pb concentration from 5 to 35 mg \kg in brome grass. EDTA has been shown not only to enhance pb desorption from the soil components to the soil solution but also to increase its transport into the xylem and its transfer from the roots to the shoots (Babu Kim and Oh 2013; Salt *et al*., 1997; Ebbs and Kochian, 1998; Epstein *et al*., 1998).

In a later study, Epstein *et al*. (1999) found that when using soil amended with 4.8 mmol/kg lead and 5 mmol/kg EDTA, the transpiration of *B. juncea* was unaffected and the concentration of EDTA and lead in shoot tissue has increased. The authors also found that a maximised lead accumulation condition by plants occurs by maximizing the concentration of lead-EDTA complex based on the EDTA extractable soil lead. In addition of organic matter amendments (e.g. compost, fertilisers and wastes) is a common practice for immobilisation of heavy metals and soil amelioration of contaminated soils. The effect of organic matter amendments on heavy metal bioavailability depends on the nature of the organic matter, their microbial degradability, salt content, soil pH and soil type (Clemente *et al*., 2005; Walker *et al*., 2003; 2004).

2.12. Phytomining

Phytomining involves the use of hyperaccumulating plants to extract valuable metals from the substrate. Hyperaccumulating plants occur naturally for many metals such as nickel, cadmium and manganese etc., where most of the metals are bio-available in soil solution for plant uptake (Baker and Brooks, 1989). This phenomenon of phytoaccumulation may also be induced in some high biomass plant species (e.g. Brassica juncea) by addition of chemicals to solubilize metals, such as gold, lead, zinc and uranium and make them available for plant uptake (Anderson et al., 1999; Robinson et al 2003). Phytoextraction has a broad application in two main areas — phytoremediation and phytomining (McGrath and Zhao,2003).

Phytomining has emerged as an environment-friendly technology that employs plants for the uptake of heavy metals (Brooks et al., 1998; Anderson et al., 1999). This technology

involves growing plants on appropriate sites, harvesting the metal-accumulating plants, and treating the biomass to recover the metal (Boominathan et al., 2004).

2.13. Related Studies to the Present Study

El-gamal (2000) investigates the distribution of heavy metal of El-Moukattam highway- Egypt. It was found that distribution of both lead and cadmium, whose levels decrease as distances from the highway increase. It also reported that traffic volume, highways layout and green barriers affect proximal levels of both lead and cadmium. Moreover, “relationships between Pb and Cd in spontaneoues vegetation and in specific bioaccumulator plants (Lolium multflorum L.) are also reported.”

Kadi (2009) also studies the effect of the heavy and light traffic on the soil composition in Jeddah city. In this study, K, As, Co, Cr, Ni, Pb, Sb, V, and Zn elemets were analysed. The findings reveal that great dependence of lead and zinc contents on traffic conditions. It also indicates that “the high zinc concentration was found along the main roads. The study suggests that the high zinc content in tested soil samples may relate traffic sources, especially vehicle tyres. “Concentrations of other elements showed little dependence on traffic conditions.”

Akbar, et al. (2006) studied heavy metal contamination in roadside soils of northern England. Samples from roadside were collected from 35 sites and analysed for four heavy metals (cadmium, copper, lead, zinc). The results revealed that lead concentration was the highest in the soil and ranged from 25.0 to 1198.0 µg/g (mean, 232.7 µg/g). Cadmium concentration was the lowest in the soil and varied from 0.3 to 3.8 µg/g (mean, 1.4 µg/g).

Nevertheless, the four heavy metals exhibited a significant decrease in the roadside soils with the increasing distance from the road.

Likewise, Aslam, et al. (2013) conducted a survey study to investigate the heavy metals contamination in roadside soil near different traffic signals in Dubai, United Arab Emirates. Seven heavy metals (i.e. Cd, Pb, Cu, Ni, Fe, Mn and Zn) were collected and analysed. It was observed that the range of lead was very high (259.66–2784.45) where there were more than two traffic signals. Similarly, the range of Pb (145.95–308.09) was also high in samples collected from the roadside having only one traffic signal. However, the range of lead at roads having no traffic signals was (8.34–58.20). Cd, Cu, Ni, Fe, Mn and Zn in soil were present within the normal range, whereas lead was reported in high concentration. The level of lead had a correlation with the traffic density attributing its origin to vehicular exhaust. This suggests that automobiles are a major source of heavy metal contamination in soil near roadsides.

In terms of phytoremediation methods, Abou-Shanab, et al. (2007) conducted a study to compare the growth and metal accumulation of *Zea mays*, *Sorghum bicolour*, *Helianthus annuus, Conyza discoridies* and *Cynodon dactylon*, which were grown in four different soils containing moderate to high amounts of heavy metals. It was concluded that "*Z. mays* and *S. bicolour* were more suitable for phytostabilization of metal contaminated soils. *Conyza* discoridies, alternatively, accumulated higher amounts of metals in their shoots."

Ghosh and Singh (2005b) conducted a research study to investigate cadmium phytoextraction ability of high biomass producing weeds in comparision to indictor plant species. The findings show that *Ipmoea carnea* was more effective in uptaking Cd from soil

than *Brassica juncea*. In addition, *I.carnea, Dhatura innoxia* and *Phragmytes karka* were the most suitable species for phytoextraction of cadmium from soil, if the all plant or above ground biomass is harvested.

It can be concluded that most of research studies mentioned above and in the literature review (see section 2.3.) reported that vehicles are the main sources of heavy metals found near roadsides. In addition, plants method was commonly used in extracting heavy metals from soils as reported in abovementioned studies and others (Wong *et al*., 2006; Ghosh and Singh 2005a*;* Schmidt *et al*., 2003; Saifullah *et al*., 2009) because it is simple technique and low in cost (see 2.6.3).

Such findings support the present study assumption in terms of heavy metal existence nearby roadsides. Moreover, these studies have encouraged the researcher for using other plants to uptake heavy metals from the soil. However, plants and amendments have been applied in this study. In addition, *Alcaligenes eutrophus* has been used for enhancing plant (*Eucalyptus*) to uptak lead from the soil, which has not been applied before.

2.14. Conclusion

The heavy metal contaminated causes some problem to the environment. One of the main sources of the heavy metal is produced by vehicles, such as emissions. These emissions produce different metal, such as Pb and Cd. It was noted that these elements decrease with the increase the distance from the road. Such results have encouraged the researcher to find ways to over-come soil contaminated by heavy metals. One of these methods is phytoremediation, which has been widely used to up-take the metal from soil because of a number of advantages, e.g. inexpensive method. In order to use this method sufficiently,

hyperaccumulator plants are recommended to use because they can up-take a large amount of heavy metals from soil. This process of up-taking heavy metal depends on heavy metal bio-availability in soil. To increase the bio-availability of metals in soil and biomass production of plants, amendments materials should be added to soils because they are used for enhancing phytoremedation of plants. Next chapter will present the investigation of the study area.

Chapter Three

Research Methodology for Soil and Plant Investigation

The aim of this chapter is to detail the methodology used and the area selected for the present study. Firstly, a brief introduction of the target city; location, climate and the select area will be highlighted. Next is a description of the methodlogy used for studing the target area, followed by the methods of identifying and preparing the plant samply. Then the results and discussion of the selected area and plant will be provided. Finally, a conclusion of the chapter will be drawn.

3.1 Materials and Methods

3.1.1. The Study Area

Azzawiyah city is located in western part of Tripoli (the capital of Libya), between 25° 00' N Latitude and 17° 00' E Longitude (Figure 3.1). The area was selected to study the affect of heavy metal in soil. The area is located in Azzawiyah city, and the selection of it was based on fact that it is very close to one of the main highways in Azzawiyah, which links Tripoli and other cities (Sabratha, Sorman Zuwarah), as well as Tunis. This fact also suggests that the target area could be exposed to heavy metals, which is emitted by vehicles (Hua *et al*. 2009; Jaradat *et al*. 1998; Elgamail 2000). The investigated site is located along a 4 Km road side. The average of vehicles used this highway was estimated between 13.000 to 15.000 vehicles in week day (7am to 7pm) the traffic, while about 8.000 vehicles are operated during holidays times (Fridays) (Azzawiyah City Council Documentary 2010). Within this area, the traffic decreases

exponentially with increasing distance away from the town centre, which is the reason for choosing this area.

Figure 3.1. Air view of Azzawiyah city in Libya

Climate

Azzawiyah has a shoreline bordering the Mediterranean Sea. The temperatures recorded in summer were 30 to 40 ºC while in winter it ranges between 17.5 to 30 ˚C. The wind speed was between 5 and 11 mph (NE), while the humidity was between 45% and 75%. The average annual rainfall in the study area has been reported to be between 0.2 mm to 0.5 mm **(**Worldweatheronline 2011).

Sampling Sites

Twenty sites were selected for this study alongside the highway road connecting Tripoli with the southern parts of Azzawiyah city. These sites cover a distance of about 4

km from Azzawiyah city centre up to Alhrshea village in the southern region of the Azzawiyah. The highway of Azzawiyah city was chosen because of its comparatively high traffic density in the country. There are many shops, building and agriculture farms located on both sides of the road. Figure 3.2 shows the place of soil samples which were collected at different distances from the edge of the main road (3 and 10 metres) on both sides; north and south of the road. The distance between each site is about 1 Km alongside the main road. It also shows the location of the heavy traffic in the target area.

Figure 3.2. Location map of the investigated area (Ministry of Agriculture. Libya 2010)

3.1.2. Soil Sampling Methodology

There are a number of methods of collecting samples, such as systematic sample, random sample and Judgement sample (Myers, 1997; Crepin and Johnson, 1993; Gilbert, 1987). In this study, systematic sample is chosen because it is suitable for the research

study. In this method, the sampling points follow a simple pattern and are separated by a fixed distance, usually in a regular grid. Locating sampling points in the field is easier with systematic sampling than with simple random sampling (Myers, 1997).

In the present study, the samples (see section 3.1) were collected from 4 km along to the road side. Topsoil samples (0-20cm) were taken at the depth of approximately 20cm deep throughout the sample sites using hand core. Then the samples were divided to 0-10 and 10-20cm. On return to the laboratory, soils samples were put it in a large container and then dried in oven at 36 $\overset{o}{C}$ for 72 hr, crushed by hand and sieved through a 4 mm stainless steel sieve to remove rocks. The samples then were analysed.

Mechanical Analysis

Soil textural analysis was carried out by using the Malvern Master Seizer 2000 analyser. Percent of sand, silt and clay were calculated.

Soil Moisture

This method provides the laboratory determination of the moisture content of a soil. It was based on removing soil moisture by oven-drying (Oven 300 Plus series). The moisture content (%) was calculated from the sample weight before and after drying. 10 g of three replicate samples was placed in foil trays, weighed and dried in the oven at 105° C for 24 h, allowed to cool and reweighed.

- Moisture Content % = (FW - DW) ÷FW × 100

  Where F W = fresh weight, D W = dry weight

Water-holding Capacity (WHC)

The percentage of water-holding capacity of soil was determined according to Alef and Nannipieri (1995). A Whatman No.2 filtered paper was placed in the bottom of a plastic pot and the mass of pot and filter paper were determined. The pot was gently filled with oven dried soil, and the mass of the pot, filter paper and dry soil sample were determined. The pot was placed into a shallow pan of water allowing only the bottom few centimetres of the pot to become wet. The soil was allowed to became saturated from the bottom of the pot to the surface. The pot was then removed from the pan of water and placed it in a humid enclosure until drainage was completed. Finally the mass of the cup, filter paper, and saturated soil sample were determined, as follows:

Mass of the dry soil = Mass of pot, filter paper, and dry soil - mass of pot and filter paper

Mass of the saturated soil = Mass of pot, filter paper, and saturated soil - mass of pot and filter paper

Mass of the water contained in the saturated soil = Mass of the saturated soil - mass of the dry soil

Percent water holding capacity = Mass of the water contained in the saturated soil ÷ mass of the saturated soil ×100

Soil pH and Conductivity

One scoop containing 10 g of air-dried soil samples (10 g scoop filled and smoothed off level without topping) was put into a 50 ml glass beaker, and 25 ml of distilled water was added. The beakers are closed and put on a shaker for 30 min at speed of

approximately 275 strokes min^{-1}. The samples were then filtered. A pH meter (Corning pH Meter 220) was used to determine pH. The pH meter was calibrated using buffer solutions of pH 4 and pH 7. The pH electrode was put in the suspension and a reading was taken after 30s. pH can be classified as the following:strongly acid (pH < 5), moderately to slightly acid (5.0 - 6.5), neutral (6.5 – 7.5), moderately alkaline (7.5 – 8.5), and strong alkaline (pH > 8.5) (Millere and Hills 2006). The same procedure of the pH experiment was used to determine EC of soil samples and an EC meter (PTI-8 Digital Conductivity Meter) was used to determine soil samples EC.

Bulk Density of soils samples

Bulk density was determined by measuring the mass of dry soil per unit of volume (g ml or g cm^{-3}) (Blake *et al*., 1986). 10 g of three replicates soil samples were placed in foil trays, weighed and dried in the oven (Oven 300 Plus series) at 80˚C for 24 h, allowed to cool and reweigh of soil samples.

Bulk Density = mass of dry soil g / total volume of soil ml

Total Organic Content (TOC)

5 g of three replicate dried soil samples were weighed accurately into a crucible and placed in a muffle furnace (Gallenkamp Muffle Furnace), and heated at 500˚C in an oven overnight and allowed to cool before being re-weighed. The loss in weight was calculated as a percentage of the initial weight of the samples. Mass of the organic matter

was determined by the mass of the soil sample before and after heating. Organic matter % = (mass of the organic matter/ mass of soil sample before heating) 100.

Total Nitrogen

Total nitrogen in soil samples was determined using the Kjeldahl method (Nelson and Sommers, 1980; Jones *et al*., 1991). Approximately 5 g of dried soil samples were mixed with 1.1g salt/catalyst mixture then placed in Kjeldahl tubes and digested with 3 ml of concentrated sulphuric acid at 350 ^{o}C for an hour and a half in a digestion block. Portions of 10 ml of H_3BO_3 indicator were added to 100-ml flask, the flask was placed under the condenser; so that the condenser tube kept embedded in the indicator solution. The digest was cooled; 20 ml of deionised water and 20 ml of 10 M NaOH were then added and been transferred immediately to the Kjeldahl distillation apparatus. The distillate was collected until the level of H_3BO_3 flask reached 70 ml. NH_3 distilled in H_3BO_3 solution was titrated using 0.01 M hydrochloric acid (HCl). The concentration of nitrogen in each sample was calculated using the following equation:

$$\text{mg N/kg dry matter} = n \times 0.01 \times 14 \times 1000/P$$

Where: N is the concentration of nitrogen in the sample.

n= ml of 0.01 N HCl p= grams of soil

Extractable Phosphorus

The extractable phosphorus from the soil samples was determined by Olsen and Sommer (1982) method. Three replicates of each of dried soil sample of 5 g were

weighed into a beaker and put into 250 ml conical flasks. To each flask, exactly 100 ml of Olsen's bicarbonate solution (4.2% w/v $NaHCO_3$, pH 8.5), were added and the flasks sealed with Parafilm. The flasks were then shaken on shakers for 30 min at 150 rpm. The mixtures were filtered into extractable bottles using Whatman no.1 filter paper and stored in a deep freezer until analysis.

5 ml aliquot of each phosphoru working standard solution was pipetted into 100 ml conical flasks. Phosphorus standard solutions, 0-10 mg l^{-1} were prepared including 0, 2, 4, 6, 8 and 10 mg l^{-1}. One millilitre of 1.5 M H_2SO_4 was added to the standard solutions, which were mixed and left to stand for 5 min, swirling the flask occasionally to release the evolved CO_2. Next, 20 ml of 12% molybdate-antimony reagent was added to all the flasks. Then 5 ml of 1.5% ascorbic acid solution was added to all flasks. The solutions were mixed and left to develop colour for 30 min. The absorbance of the solutions was determinated at 880 nm (Spectrophotometer CE1010).

Extractable Potassium, Sodium, Calcium and Magnesium

5 g of three replicates of dried soil sample were weighed into a beaker and placed in 100 ml conical flasks. Fifty millilitres of 1 M ammonium nitrate was added to the samples and blank. At the same time, three spikes were prepared by measuring 0.05 ml of 1000 mg l^{-1} from each stock solution into 50 ml, which gave a concentration of 1 mg l^{-1}. The samples were then shaken for 1 h at 250 rpm using an electronic shaker, and filtered using the Whitman No.1 filter papers. All the samples were analysed by ICP AES.

Total heavy Metal Content

The total heavy metals were determined using acid digestion of the samples in HNO_3 followed by ICP-AES analysis to quantify the elements. In practice,1g of three replicates of dried soil samples were weighed into a beaker and placed in digestion tubes. Five millilitres of 98% HNO_3 was added to samples, spikes and blank. The mixture was heated to 180˚C for 2 h until the solution became clear, with digested mineral matter settling out, and then allowed to cool, and filtered through a 125 mm Whatman No. 1 filter paper. The solution was then diluted to 100 ml with deionised water and than analysed by ICP-AES.

Extractable Heavy Metals

The single extraction method using $CaCl_2$ was chosen for this study because it is useful for evaluating the mobility and bioavailability of these trace elements in urban soils (Novozamsky, et al. 1993; Hammer and Keller 2002, Pueyo *et al.* 2003. 5g of three replicates dried soil samples were weighed into a beaker and placed in extractable conical flasks. Fifty millilitres of 0.01 M $CaCl_2$ solution were added to samples, spikes and blank. The samples were then shaken for 2 h at 140 rpm using an electronic shaker and then filtered through a 125 mm Whatman No. 1 filter paper. The solution was then diluted to 50 ml with deionised water. All the samples were measured by ICP AES.

3.2. Concentrations Ratios of some Trace Metals in Soils, Soil Solution, and Plants

As shown below, table 3.1 summarises the concentrations of trace metals in soils and plants reported in different studies. These results have been used in this research.

Table 3.1. Typical concentrations of some trace metals in soils, soil solution, and in plants

References: [a]Swaine (1955); [b]Kabata-Pendias and Pendias (1984); [c]Allaway (1968); [d]Ministry of Housing, Spatial Planning and Environment (2007).

3.3 Plant Sampling, Identification and Preparation

The collected plant species were identified according to the location from which they were collected in Azzawiyah city. Figure 3.3 shows the plant sampling locations in the study area. A whole plant was excavated and divided into roots and shoots, then, both

plant parts were thoroughly washed several times using distilled water. The washed plant material was then dried at 70^{o}C for 72 h and was grounded to pass through a 2-mm mesh sieve. 400 mg of dry plant tissue were digested in HNO_3 (Huang *et al*., 1997) and then were brought to a constant volume with deionised water. Digests were analysed for Co, Cr, Cu, Ni, Fe, Pb, Cd, and Zn using ICP–AES.

Figure 3.3: Plant sampling locations in the study area.

3.4. Statistical Analyses.

Data was analysed using Minitab software (Version 16 English), the variance was tested in significant results between variables (ANOVA) separated using a Tukey test where $p \leq 0.05$

3.5. Results and Discussion:

3.5.1. Physical and Chemical Characteristics of Soil Samples

The behaviour of trace metals in soils depends not only on the level of contamination (as expressed by the total content), but also on the form and the origin of

the total metal, as well as the properties of the soils (Tessier and Campbell 1988; Chlopecka *et al*., 1996). Physical and chemical properties of the soil have a direct influence on the form of the metal contaminant, its mobility, and the technology selected for remediation (Gerber *et al*., 1991).

The findings show that all soils were granular with sandy texture (table 3.2). Sandy soils are generally poor in nutrient reserves and have low WHC (Gomes *et al*., 2003; Sanders, 1983). The moisture content and water holding capacities in soil samples were generally as low as in the range from 0.1 to 0.5% and from 7.40 to 9.41%, respectively. Soil pH values were generally in the alkaline range (e.g. 7.5 - 8.5). This means that soil pH is one of the most influential parameters controlling the conversion of metals from the immobile solid-phase to more mobile and/or bioavailable solution phase (Sanders, 1983). The solubility of heavy metals is generally greater in the normal agriculture soils pH range (e.g. approximately pH 5.0 to 7.0) (Sanders, 1983). While the concentration of organic matter (OM) content varied between 0.05% and 0.09%. Such findings indicate that OM affects crop growth either directly by supplying nutrients or indirectly by modifying soil physical properties, such as stability of aggregates and porosity that can improve the root environment and stimulate plant growth. Moreover, OM and pH are the most important factors that control the availability of heavy metals in the soil (Karaca, 2004; Darwish *et al*., 1995).

Table 3.2: The mean of chemical and physical properties of the collected soil samples.

Site No.	Sand	Silt	Clay	OM	WHC	MC	EC (mmhos.cm^{-1})	pH	BD g ml^{-1}
	……..…….%.....								
1	93.45	2.30	4.25	0.09	8.90	0.4	1.6	7.71	1.64
2	93.82	2.42	3.76	0.08	9.01	0.3	1.2	7.98	1.61
3	91.81	2.70	5.49	0.08	8.65	0.1	1.8	7.82	1.71
4	92.56	2.35	5.09	0.08	8.55	0.3	1.9	7.78	1.69
5	90.22	3.55	6.23	0.06	9.31	0.3	1.0	7.79	1.58
6	91.70	2.70	5.60	0.06	9.25	0.3	0.9	8.10	1.59
7	89.15	3.40	7.45	0.07	7.89	0.2	1.0	7.81	1.48
8	90.11	3.50	6.39	0.06	8.65	0.1	0.6	7.50	1.50
9	93.80	2.45	3.75	0.09	9.12	0.1	1.1	7.86	1.57
10	93.76	2.44	3.80	0.08	8.78	0.2	1.0	8.50	1.55
11	92.60	2.40	5.00	0.07	7.77	0.2	1.2	8.00	1.68
12	91.86	2.75	5.39	0.05	8.21	0.2	1.0	7.98	1.62
13	91.79	2.71	5.50	0.05	7.40	0.3	1.7	7.80	1.63
14	92.62	2.34	5.04	0.07	8.11	0.5	1.6	7.78	1.61
15	91.65	2.63	5.72	0.06	7.94	0.4	1.0	8.00	1.60
16	89.98	3.50	6.52	0.08	7.56	0.3	1.1	7.85	1.35
17	90.20	2.30	7.50	0.08	8.25	0.2	0.6	7.62	1.41
18	91.10	2.23	6.67	0.06	9.41	0.3	0.9	7.90	1.60
19	91.35	2.10	6.55	0.06	9.01	0.4	1.0	8.24	1.45
20	91.89	2.34	5.77	0.05	7.68	0.3	1.2	7.98	1.65

WHC = Water holding capacity, MC = Moisture Content, OM= Organic Matter, BD = Bulk Density and EC = Conductivity.

In terms of the cation exchange capacity (CEC), the findings (Table 3.3) show that most of the soil samples were ranged from 7.63 to 9.10 meq 100g^{-1} soil. The CEC of

soil is also of major importance in determining the extent to which heavy metals are adsorbed by solid phase constituents, hence, the extent of their solubility.

Table 3.3: The mean of chemical properties of soil samples

Site number	N	P	Ca^{++}	Mg^{++}	K^{+}	Na^{+}	CEC
	…. mg kg^{-1}…		……..meq 100 g^{-1}……				
1	0.14	0.70	6.70	0.89	0.20	0.30	8.09
2	0.13	0.70	5.79	1.15	0.17	0.28	7.35
3	0.10	0.80	6.10	1.14	0.20	0.32	7.76
4	0.14	0.75	7.02	1.04	0.19	0.19	8.44
5	0.11	1.10	7.50	1.10	0.18	0.26	9.04
6	0.12	0.80	7.30	1.08	0.15	0.23	8.76
7	0.20	1.10	6.79	0.91	0.20	0.28	8.18
8	0.19	1.15	7.54	1.21	0.23	0.34	9.31
9	0.19	1.11	6.52	1.01	0.22	0.29	8.04
10	0.11	0.90	7.55	1.10	0.23	0.26	9.10
11	0.10	0.75	6.22	0.96	0.17	0.32	7.67
12	0.08	0.85	6.60	0.85	0.15	0.33	7.93
13	0.09	0.82	6.08	1.11	0.20	0.30	7.69
14	0.11	0.90	6.91	1.10	0.30	0.26	8.57
15	0.09	0.86	6.04	1.12	0.19	0.28	7.63
16	0.18	1.12	6.64	1.09	0.20	0.30	8.23
17	0.19	1.14	7.43	0.98	0.20	0.31	8.92
18	0.15	0.90	6.08	1.09	0.25	0.40	7.82
19	0.10	0.89	6.09	1.10	0.21	0.30	7.70
20	0.10	0.80	5.91	0.98	0.30	0.45	7.64

(CEC) = cation exchange capacity

In general, soils with high CECs can adsorb larger amounts of heavy metals than soils with low CEC. Soil organic matter also has a high specific surface area; consequently the majority of CEC in soil is from organic matter (Abou-Shanab *et al*., 2007). Table 3.3 shows the total concentrations of P, N, Ca, Na, K, and Mg were low. Ion-exchangers have been extensively used to assess soil macronutrient availability in plants, mostly for P, but also for Ca, Mg, K, N, and S (Qian *et al*., 1992).

3.5.2. Total and $CaCl_2$ Extractable Metal Concentration

The findings show that the highest concentrations of Co element was 22.20^{cde} mg kg^{-1} in the sample No. 19 (10mN) at depth 0-10 cm and the lowest concentration was 4.05^{g} mg kg^{-1} in the sample No. 11 at the depth 10-20 cm, while the P-value was < 0.01 and the Co extractable value of sample No. 19 (10mN) was 0.27^{cde} mg kg^{-1} (see Table 3.4a and appendix1)

Table 3.4a. The mean of total and extractable of Co and Cr at different sites.

Site	Profile	Co mg kg^{-1}				Site	Profile	Cr mg kg^{-1}			
		T	P-value	E	P-value			T	P-value	E	P-value
11	10-20	4.05^{g}	0.22	0.05^{a}	< 0.01	3 (3mS)	0-10	63.12^{a}	< 0.01	0.57^{f}	< 0.01
19(10mN)	0-10	22.20^{cde}	< 0.01	0.27^{cde}	< 0.01	11	10-20	28.11^{c}	< 0.01	033^{f}	< 0.01

a , b ,c , d ,e mean don't share the same letter are significantly different.

The highest concentration of Cr element was 63.12^{a} mg kg^{-1} in the sample 3 (3mS), and the lowest concentration was 28.11^{c} mg kg^{-1} in the sample No. 11 at the depth 10-20 cm, while the P-value was < 0.01 and the Cr extractable value of sample No. 3 (3mS) was 0.57^{a} mg kg^{-1}. The findings presented in Table 3.4b shows the highest concentration of Cu element which was 50.71^{a} mg kg^{-1} in the sample No. 3(3mS) at depth 0-10 cm and the lowest concentration, which was 24.30^{abc} mg kg^{-1} in the sample No. 20(10mN) at the depth 10-20 cm. The P-value of this element (sample 20 "10mN") was < 0.01 and the Cu extractable value of the same sample was 0.30^{cde} mg kg^{-1} (see also appendix 1 for more details). Table 3.4.b also presents the highest and the lowest concentration of Ni element in the target area of the study. It reveals that 20.71^{abc} mg kg^{-1} in the sample 2 (10mS) was the maximum concentration of Ni in the soil, whereas the P-value of the same sample was < 0.01 and the extractable value was 0.26^{abc} mg $kg^{-1.}$ In terms of the lowest concentration of the Ni element, 14.03^{efg} mg kg^{-1} in the sample No. 16 (10mN) at the depth 10-20 cm was the lowest concentration, whereas < 0.01 was the P-value and 0.29^{g} mg kg^{-1} was the extractable value in the same sample; No. 16 (10mN).

Table 3.4b The mean of total and extractable Cu and Ni amounts at different sites

Site	Profile	Cu mg kg^{-1}				Site	Profile	Ni mg kg^{-1}			
		T	P-value	E	P-value			T	P-valu	E	P-value
3(3mS)	0-10	50.71^{a}	< 0.01	0.62^{a}	< 0.01	2 (10mS)	0-10	20.71^{ab}	< 0.01	0.26^{abc}	< 0.01
20(10mN)	10-20	24.30^{abc}	< 0.01	0.30^{cde}	< 0.01	16(10mN	10-20	14.03^{ef}	< 0.01	0.29^{g}	< 0.01

a , b ,c , d ,e mean don't share the same letter are significantly different. T= total metal, E =extractable metal

Table 3.4.c presents the highest and lowest concentrations of Fe element of the study area. It shows that 1897.11[a] mg kg^{-1} in the sample 9(3mN) was the highest concentration of Fe in the soil. The P-value of the same sample was < 0.01 and the extractable value was 19.76[a] mg kg^{-1}. The lowest concentration of the Fe element was 824.39[a] mg kg^{-1} in sample No. 19 at the depth 10-20 cm, whereas 0.344 was the P-value and 8.66[b] mg kg^{-1} was the extractable value in the same sample No.19.

The findings in Table 3.4c also illustrate the highest and the lowest concentrations of Cd element. The highest concentration was 0.64[a] mg kg^{-1} in the sample No. 1(3mS) at depth 0-10 cm, whilst the lowest concentration of this element was 0.10[bcbe] mg kg^{-1} in sample No. 20 at the depth 10-20 cm. The P-value of this element (sample 20) was estimated to be < 0.01 and the Cd extractable value of the same sample was ND (see also appendix 1 for more details).

Table 3.4c The mean of total and extractable of Fe and Cd at different sites

Site	Profile	Fe mg kg^{-1}				Site	Profile	Cd mg kg^{-1}			
		T	P-value	E	P-value			T	P-value	E	P-value
9(3mN)	0-10	1897.11[a]	< 0.01	19.76[a]	< 0.01	1(3mS)	0-10	0.64[a]	< 0.01	ND	
19	10-20	824.39[a]	0.434	08.66[b]	< 0.01	20	10-20	0.10[bcdef]	< 0.01	ND	

a , b ,c , d ,e samples mean don't share the same letter are significantly different. T= total metal, E =extractable metal

The findings presented in Table 3.4d shows that the highest concentration of Pb element was 840.40^{a} mg kg^{-1} (sample No. 1(3mS) at depth 0-10 cm), the P-value was < 0.01 and the extractable value was 8.80. The lowest concentration of Pb was recorded as 48.895^{i} mg kg^{-1} in sample No. 15 (10mS) at the depth 0-10 cm. The P-value of this element was < 0.01 and the extractable value was 0.51^{i} mg kg^{-1} (see Table 3.4.d and also appendix 1 for more details).

The highest and the lowest concentrations of Zn are also presented in Tabel 3.4d. The table shows that 98.50^{a} mg kg^{-1} concentration in sample 1 (3mS) was the highest concentration of Zn element in the soil, whilst the P-value of the same sample was < 0.01 and the extractable value was 1.20^{a}. 40.08^{cd} mg kg^{-1} in sample No. 16 at the depth 10-20 cm was the lowest concentration of the Zn element, whereas < 0.01 was the P-value and 0.50^{dc} was the extractable value in the same sample No. 16.

Table 3.4d The mean of total and extractable of Pb and Zn at different sites

Site	Profile	Pb mg kg^{-1}				Site	Profile	Zn mg kg^{-1}			
		T	P-value	E	P-value			T	P-value	E	P-value
1(3mS)	0-10	840.40^{a}	< 0.01	8.80^{a}	< 0.01	1(3mS)	0-10	98.50^{a}	< 0.01	1.20^{a}	< 0.01
15(10mS)	0-10	48.895^{i}	< 0.01	0.51^{i}	< 0.01	16	10-20	40.08^{ed}	< 0.01	0.50^{dc}	< 0.01

a , b ,c , d ,e mean don't share the same letter are significantly different.T= total metal, E =extractable metal

The total metal content is important because it determines the size of the metal pool in the soil and thus that available for metal uptake (Ibekwe *et al*., 1995). Therefore, the soil samples were analysed for total and $CaCl_2$ extractable concentrations of Co, Cr, Cu, Ni, Fe, Cd, Pb, and Zn. The obtained results presented in Table 3.3 showed that each site exhibited a high concentration of one or two metals. Variation was also reported in the extractable metal content, i.e. biologically available metals in comparison with the total metal content in the same soil. This can be attributed to the behaviour of trace metals in soils that depends not only on the level of contamination, as expressed by the total content, but also on the form and origin of the metal and the properties of the soils themselves (Chlopecka *et al*., 1996).

In this experiment, the bioavailable concentrations of metals extracted by 0.5 M $CaCl_2$ extraction were found to be lower than the total concentration of these metals. This can be attributed to many factors affecting trace element availability in soils including physical and chemical properties (Alloway 1995; Pueyo *et al*., 2003).

The highest concentration values of metals were recorded in soil samples collected at 3 m south and north of the roadside. Lead, the element of most concern in environmental heavy metal pollution, exhibited high levels of contamination closer to highway. This fact can indicate that the contamination level of lead decreases with increasing distance away from the highway (Table 3.4.d and appendix1). Whereas unchanging levels would show that the heavy metal concentrations were as a result of a function of the soil structure. Since the fuel used by automobiles in Libya is mostly

leaded, the source of such contamination is most properly was due to the lead particulate matter emitted from vehicles, which settles not far from the highway (Harrison and Laxen, 1981). As the distance from the road increases, the Pb level decreases. The maximum Pb concentration (840 mg kg^{-1}) was detected in soil samples collected at 3 m south of the road. Therefore, the lead contamination of soil was restricted to short distances from both sides of the highway. However, some investigators have found that lead contamination of soil may reach 100 m from the main road but the high contamination within 10 m from both roadsides (Jaradat *et al*., 1998; Elgamail, 2000 and Hua *et al*., 2009).The major heavy metal detected in most polluted soils was Pb (840 mg kg^{-1}) at depths (0-10 cm), which was higher compared with those values of Pb published earlier (Table 3.1).

Zinc, in the soil next to the highway, exhibited elevated levels (e.g. 67 mg kg^{-1} 3 m south of the road). This value is relatively small compared with many other studies (Hewitt and Candy 1990; Culbard *et al*., 1988). In this study, the Pb : Zn ratio in soil was greater than unity, which may indicate soil-lead pollution has been caused by automobiles. Similar results were found by other investigators (Ho and Tai 1988; Culbard *et al*., 1988). However, some reports (Hewitt and Candy 1990; Davies, 1984) found a ratio of less than unity, which was related to the local weather and soil conditions. Since no major industry exists in the study areas, such as smelting operations, it may be assumed that the primary sources of Zn are probably the attrition of motor vehicle tyre rubber exacerbated by poor road surfaces, and the lubricating oils in which Zn is found as

a part of many additives such as zinc dithiophosphates. Co, Cr, Ni and Cd, on the other hand, exhibited lower levels of contamination than those of other studies (Davies *et al*., 1985; Culbard *et al*., 1988). The findings also show that the concentration values of these metals were higher than those values generally observed in agriculture soils, which can be considered as toxic (Table 3.1).

Table 3.5. The Mean of total metals concentration at different depths of soil samples.

	Element concentration mg kg^{-1}							
Depth	**Co**	**Cr**	**Cu**	**Ni**	**Fe**	**Cd**	**Pb**	**Zn**
0-10 cm	10.85^{a}	46.15^{a}	32.38^{a}	18.62^{a}	1442.10^{a}	0.30^{a}	410.58^{a}	75.27^{a}
10-20cm	5.53^{a}	37.16^{b}	30.12^{b}	17.44^{b}	1156.12^{b}	0.17^{a}	223.39^{b}	44.50^{b}
P-Value	0.51	0.01	0.00	0.01	0.03	0.07	0.02	0.03

a, b, Means within rows with the same litter do not differ significantly (ANOVA, $p < 0.05$)

Based on the result shown in Table 3.5, the mean values of (10.85, 46.15, 32.38,18.62, 1442.0.30, 410.58, and 75.27 mg kg^{-1}) of total CO, Cr, Cu,, Ni, Fe, Pb, and Zn, respectively, in soil collected from the top surface (0-10 cm depth) were statistically higher compared with the soil profile in 10-20 cm depth.

3.5.3 Comparison and Classification with Guideline Concentrations

The concentrations of several major metal contaminants in Azzawiyah soils were compared with other concentration values: ICRCL 59/83 trigger concentration values (Inter Departmental Committee for the Redevelopment of Contaminated Land 2007),

Dutch action values (Ministry of Housing, Spatial Planning and Environment 2007) and guidance, which are given in Table 3.1 and Figure 3.4. Only the highest concentration of Pb was compared with above mentioned values (see Figure 3.4.).

The Pb concentration in soil sample (e.g. sample1) at depths 0-10cm was found to be higher than the ICRCL 59/83 threshold value trigger concentrations, the Dutch action guidelines and the guidelines shown in Table 3.1. Therefore, these levels are used for screening purposes. When the guidelines are exceeded, some action should be taken, as it involves monitoring of the area, and remediation programmes must be established. The contamination of soil by Pb is mainly related to human activities. Soil remediation is required to reduce risk to humans or the environment from toxic metals.

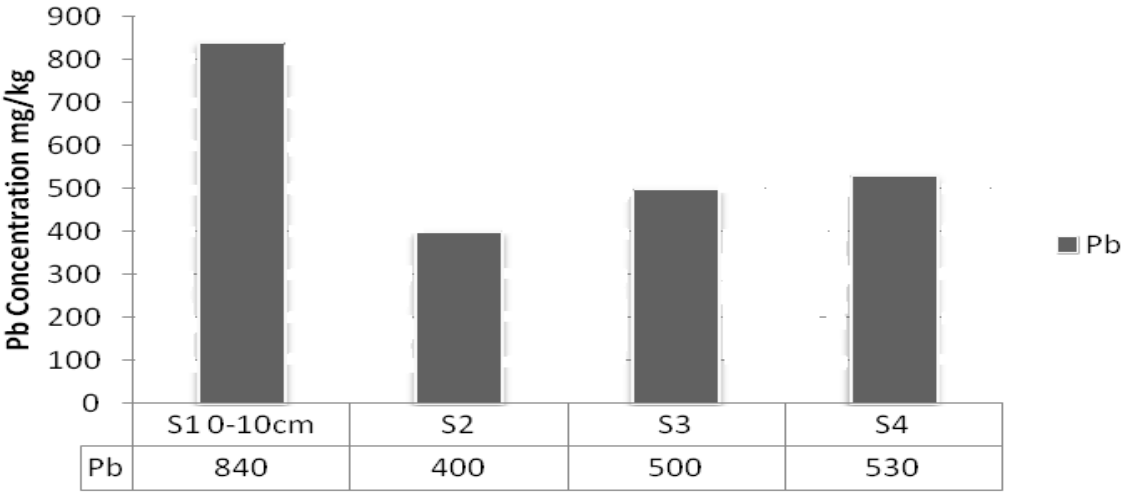

Figure 3.4: Concentrations of Pb element in Azzawiyah area (e.g S1 Sample1) compared with, S2= Concentration in soil considered toxic (Kabata-Pendias and Pendias (1984); S3=Threshold value (ICRCL trigger value), and S4=Action value (Dutch list)

Lead elements in the Soil Samples

Distribution of Pb between various distances and depths results from the large amounts of emission of heavy metal particles from the vehicles exhausts in the Azzawiyah area. It can be noticed that the concentration of lead element decreases as the distance increases away from the traffic area as shown in Figures 3.5 and 3.6.

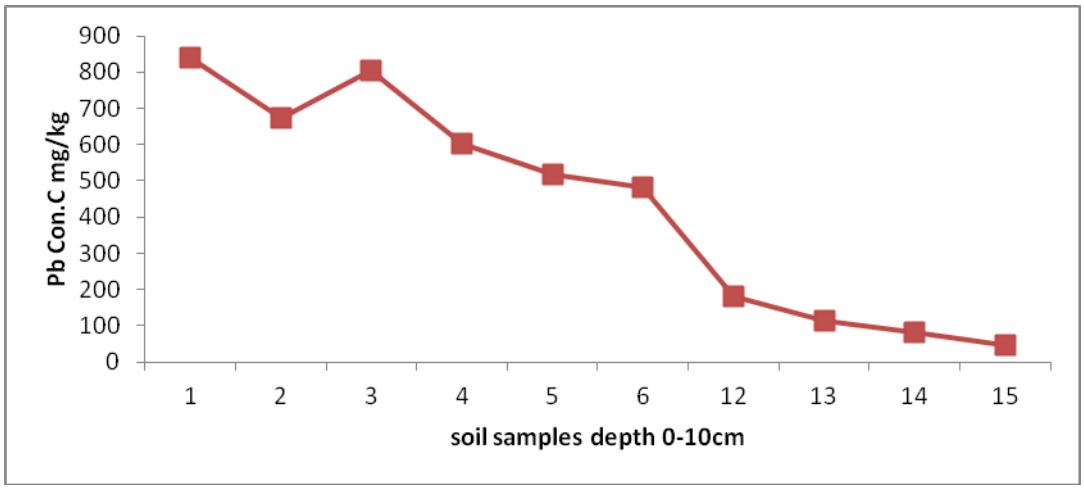

Figure.3.5. Average concentration of Pb across the south sample site

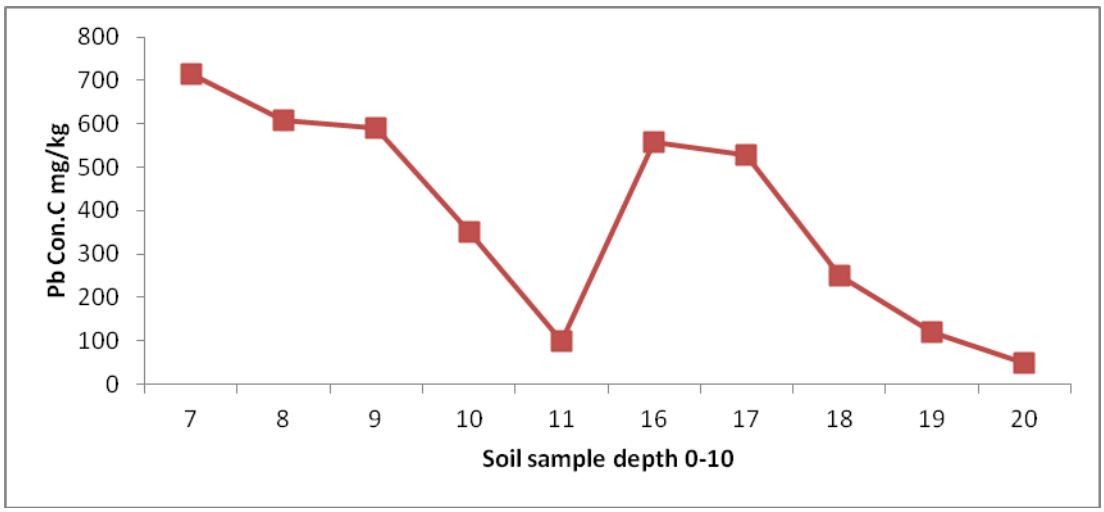

Figure.3.6. Average concentration of Pb across the North sample site.

3.5.4 Results and Discussion for plant Analysis

Doedonea viscose was collected from the sites of investigation. This plant species was dominant on the south and north of the roadside. The findings in Table 3.6 shows that concentration of Pb in *Doedonea viscose* root sample was 48.0 mg kg^{-1} at the sample No 1 SC*, while the Pb concentration in the shoot sample at the same sample was 12.1 mg kg^{-1}. Whereas the Pb concentration in *Doedonea viscose* root in the sample 2 SW** was 12.2 mg kg^{-1}. The Pb concentration in the shoot in the same sample was 4.1 mg kg^{-1}.

In terms of Zn concentration in the root in sample 1 SC* was 29.30 mg kg^{-1}, whilst the concentration of the same element in the shoot was 17.90 mg kg^{-1}. The concentration of Zn in another sample in the plant root was 31.20 mg kg^{-1}, while concentration in the shoot was 9.0 mg kg^{-1}. The concentrations of the other elements in both roots and shoots were lower than Pb and Zn. Moreover, Cd element concentration was undetectable in *Doedonea viscose* plant.

Table 3.6. The mean of heavy metal concentrations in *Doedonea viscose* collected from different sites.

Location	Pb		Zn		Cd		Fe		Ni		Cu		Cr		Co	
	mg kg^{-1} dry wt															
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
1 SC*	48.0	12.1	29.3	17.9	ND	ND	520	235.1	ND	ND	18.1	2.9	12.1	2.1	ND	ND
2 SW**	12.2	4.1	31.2	9.0	ND	ND	324.9	118.2	ND	ND	7.9	2.1	3	ND	ND	ND

* SC = South and close to the roadside which contains (840, 67, 0.6, 1092, 3, 26, 11, and 2 mg Kg^{-1} dry soil) of total Pb, Zn, Cd, Fe, Ni, Cu, Cr, and Co, respectively; ** SW = South and away from the roadside (Agriculture field) contains 30, 53, 4, 875, 7, 18, 6, and 12 mg Kg^{-1} dry soil) of total Pb, Zn, Cd, Fe, Ni, Cu, Cr, and Co, respectively. S = shoot and R = Root

The normal ranges of metal concentrations in plants are 0.03-15 mg Cr kg^{-1}; 4-15 mg Cu kg^{-1}; 0.1-10 mg Pb kg^{-1}; 0.02-5 mg Ni kg^{-1}; 0.05-0.5 mg Co kg^{-1}; 0.2-0.8 mg Cd kg^{-1} and 8-400 mg Zn kg^{-1} (Reeves *et al*., 1995; Kabata-Pendias and Pendias 2001).

Total metal content is important because it determines the size of the metal pool in soil and thus available for metal uptake (Ibekwe *et al*., 1995). Results show that the metal content in plant tissues collected from the site close to the road side was relatively high compared with the same plant species collected from the agricultural field (Table3.5).

Hyperaccumulation is defined as uptake and sequestration of exceptionally high concentrations of an element in the above ground parts of a plant under field conditions. Baker and Brooks, (1989) argue for the recognition of standard criteria for hyperaccumulation at concentrations of 10,000 mg kg^{-1} for Mn or Zn; 1,000 mg kg^{-1} for Co, Cr, Cu, Pb, and Ni; and 100 mg kg^{-1} for Cd. The results obtained from *Doedonea viscose* indicated that there was no Cr, Cu, Pb, Ni, Co, Cd, and/or Zn hyperaccumuators according to Brooks (1983), and Baker *et al*., (2000).

3.6. Conclusion

The result of this study generally revealed that the physical and chemical characteristics of soil samples were poor. Furthermore, all the soil samples were sandy, organic matter was poor and CEC were low in all samples. In terms of the soil close to the roadsides was contimenated by heavy metals, especially with Pb elements. The level

of Pb found in this study was higher compared with other studies mentioned in section 3.5.3.

Accumulation of metal in the soil and subsequent transfer to plants growing along the edge of the road could occur as a result of continual usage of the road by automobiles. The greater concentrations in soils near the roadside represent long term contamination of heavy metal from transport in a roadside environment. Examining the lead content of roadside soil, it can be concluded that lead generally decreases with increasing distance from the roadsides. Also the results shows that the *Doedonea viscose* was not hyperaccumuators plant for Cr, Cu, Pb, Ni, Co, Cd, and Zn. Consequently, the following chapter will present the alternative plants that could accumulate metal from the contaminated soil.

Chapter Four

The Uptake of Metals in Soil by Plants under Controlled Condition

This chapter, first, illustrates the materials and methods used in the pot experiment. Then the results of the phytoremediation are presented. Finally, discussions of the findings are presented and a conclusion of the chapter is drawn.

4.1. Introduction

The use of plants for the rehabilitation of polluted environments is known as phytoremediation. Phytoremediation, as mentioned in Chapter 2 section 2.6.2, is a method used to remove metals from soil. It is also used to recover the metals concentrated in the aboveground portion of the biomass by harvesting and examining the biomass (Schnoor, 1997). The choice of an appropriate plant species is an important stage for the successful application of phytoremediation techniques (Baker *et al.* 1994, Brooks 1998).

This technology has been developed after the identification of certain plants "hyperaccumulators".These plants have several beneficial characteristics, such as the ability to accumulate metals in their shoots and an exceptionally high tolerance to heavy metals. The accumulation of high amounts of metals from soil and transfer it to plants can make hyperaccumulators suitable for phytoremediation purposes (Baker *et al*., 2000; Chaney, 1983; Ernst, 1996; Abou-Shanab *et al*., 2007; 2008).

A few plants species, such as “pseudometallophytes” and “absolute metallophytes” are able to survive and reproduce on soils heavily contaminated with Zn, Cu, Pb, Cd, Ni, Cr, and As (Baker, 1987). “Pseudometallophytes” grow on both contaminated and non-contaminated soils, whereas the “absolute metallophytes” grow only on metal-contaminated and naturally metal-rich soils (Baker, 1987). Depending on the plant species, metal tolerance may result from two basic strategies: metal exclusion and metal accumulation (Baker, 1981; Baker and Walker, 1990). The exclusion strategy is usually used by "pseudometallophytes", which include avoidance of metal uptake and restriction of metal transport to the shoots (De Vos *et al*., 1991).

In this study, *Indian mustard, Medicago sativum* and *Eucalyptus camaldeulensis* plants were used to remove the metals from the soil as described in Chapter 2. These plants were chosen, as they have the ability to transport metals from roots to shoots. This process was undertaken in a greenhouse experiment, which will be descriped in the following section.

4.2. Materials and Methods

4.2.1. Pot Experiment

Soil Sources Characterisation and Preparation

Soil samples were collected from sites chosen for their traffic activities as mentioned earlier (Chapter 3, Section 3.1). Soil samples were thoroughly mixed in large

containers and air-dried at room temperature, and then sieved to remove rocks and undecomposed organic materials. Physicochemical characteristics of soil were determined as mentioned earlier, and are given in Table 3.1.

Pot Experiment

A greenhouse experiment was conducted to evaluate three-plant species in terms of accumulation of heavy metals. To initiate the experiments, 2 kg of soil were placed into plastic pots (18 cm in diameter and 13 cm in depth). Plant species (*I. mustard, M. sativum*, and *E. camaldeulensis*) were chosen for this study based on their high biomass, as well as their ability to remove heavy metals from contaminated sites (see section 2.8). Seeds of *Indian mustard, Medicago sativum* and *Eucalyptus camaldeulensis* were sown in plastic pots which contains Pb contaminated soil (840mg/kg) and uncontaminated soil (control) with four replicates for each treatment. The experiment was carried out in a greenhouse illuminated with natural light at 25 ^{o}C. After germination, the seedlings were thinned to two plants per pot and grown for 90 days. The pots were watered with distilled water throughout the experiment. The experiment was replicated three times. Plants were collected at the end of the experiment.

Plant Harvest and Analysis

After 90 days, plants were gently removed from the pots. Shoot and roots were separated and the weights of both were measured. Plant shoots and roots were washed with deionised water, rinsed, and dried in oven at 70^{o}C, and the dry matter (DM) was also

measured. Plant materials were ground and two grams of milled plant matter was digested with a mixture of HCl/ HNO_3 (4:1, v/v) for two hours. Digests were analysed for Co, Cr, Cu, Ni, Pb, Cd, and Zn using ICP–AES, as described in section 3.2.10

4.3. Statistical Analyses

Data was analysed using Minitab software (Version 16 English), the variance was tested in significant results between variables (ANOVA) separated using a Tukey test where $p \leq 0.05$

4.4 Phytoremediation Results

4.4.1 The Effect of Soil Contamination on the Fresh Root Weight

The effect of soil contamination on the fresh root weight of *Eucalyptus camaldeulensis, Indian mustard* and *M. sativum* seedlings is shown in Figure 4.1. Two–way analysis of variance indicates that there was a significantly differentweight after the effect of soil contamination on the plant species seedlings as a single variable. The interaction between plant species and soil contamination on the fresh root weight of seedling was also significant.

In both the uncontaminated and the contaminated soils, a greater fresh weight of root was observed in *Indian mustard* followed by *Eucalyptus camaldeulensis*. Moreover, irrespective to plant species, the weight of fresh root seedlings was lower in those grown in contaminated soils than for those grown in uncontaminated ones.

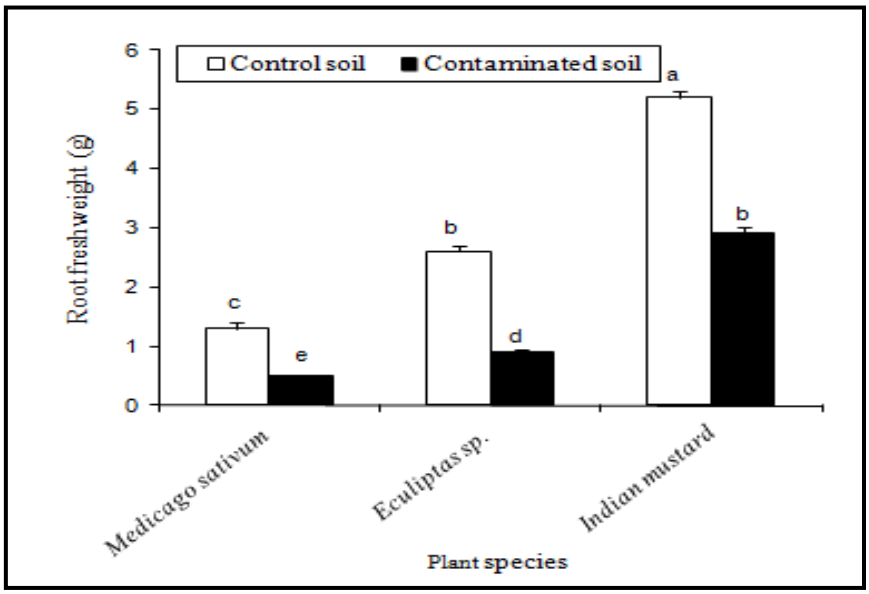

Figure 4.1: Fresh root weight of plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P \leq 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

4.4.2 The Effect of Soil Contamination on the Fresh Shoot Weight

The effect of soil contamination on the fresh shoot weight of *E. camaldeulensis*, *I. mustard* and *M. sativum* seedling is shown in Figure 4.2. Two-way analysis of variance indicated that there was a significant effect of plant species and soil contamination on the seedling fresh shoot weight as a single variable. The interaction between plant species and soil contamination on the seedling fresh shoot weight was also significant.

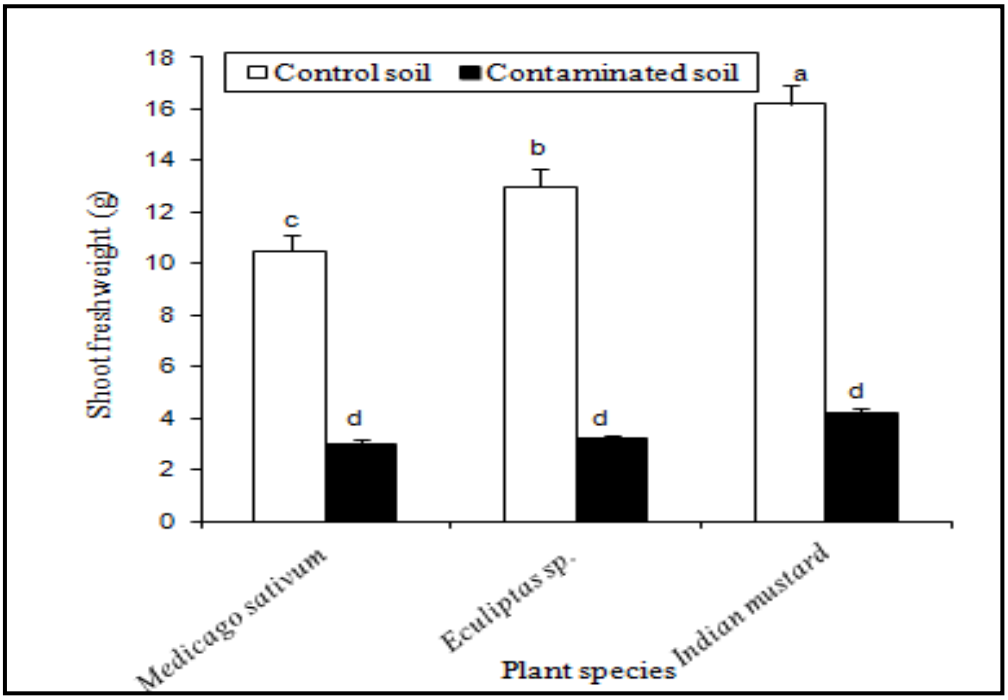

Figure 4.2: Fresh Shoot weight of plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

In the uncontaminated soils, the seedling fresh shoot weight was greater for *Indian mustar*d than that for *Eucalyptus camaldeulensis*, and is also greater than that for *M. sativum*. However, in contaminated soils and irrespective of species, the seedling fresh shoot weigh did not vary significantly.

4.4.3. The Effect of Soil Contamination on the Dry Root Weight

Figure 4.3 presents the effect of soil contamination on the seedling dry root weight of the *E. camaldeulensis*, *I. mustard* and *M. Sativum*. In this experiment, the two–way analysis of variance has indicated that there was a significant effect of plant species and soil contamination on the seedling dry root weight as a single variable. The interaction between plant species and soil contamination on the seedling dry root weight was also significant.

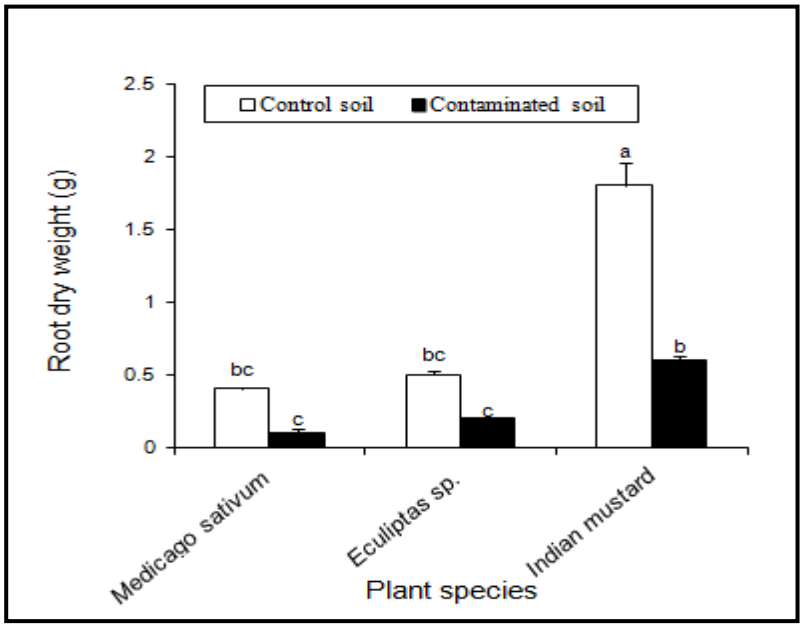

Figure 4.3: Dry root weight of plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different P < 0.05. *Indian mustard =Brassica juncea*). *a , b ,c , d ,e mean don't share the same letter are significantly different.*

In uncontaminated soils, the dry weight of seedlings root was greater with *Indian mustard* than that for both *Eucalyptus camaldeulensis* and *Medicago sativa*, which were similar. However, in the contaminated soils experiment and irrespective of species, the root dry weight of the obtained seedlings did not differ significantly.

4.4.4 The Effect of Soil Contamination on the Dry Shoot Weight

The effect of soil contamination on the seedling dry shoot weight of *Eucalyptus camaldeulensis, Indian mustard* and *M. Sativum* is shown in Figure 4.4. Once more, the two-way analysis of variance has indicated that there was a significant effect of plant species and soil contamination on the seedling dry shoot weight as a single variable. The interaction between plant species and soil contamination on the seedling dry shoot weight has also been significant.

In uncontaminated conditions, the amount of dry weight of shoot was observed to be greater in *Indian mustard*, followed by *Eucalyptus camaldeulensis*, and lastly in *M. Sativum*. In regard to the seedling dry shoot weight of plants grown in contaminated soils, it was observed that Indian mustard exhibited greater weight than that for the other two species. However, the dry shoot weight of *Eucalyptus camaldulensis* and *Medicago sativum* seedling has shown similar amounts.

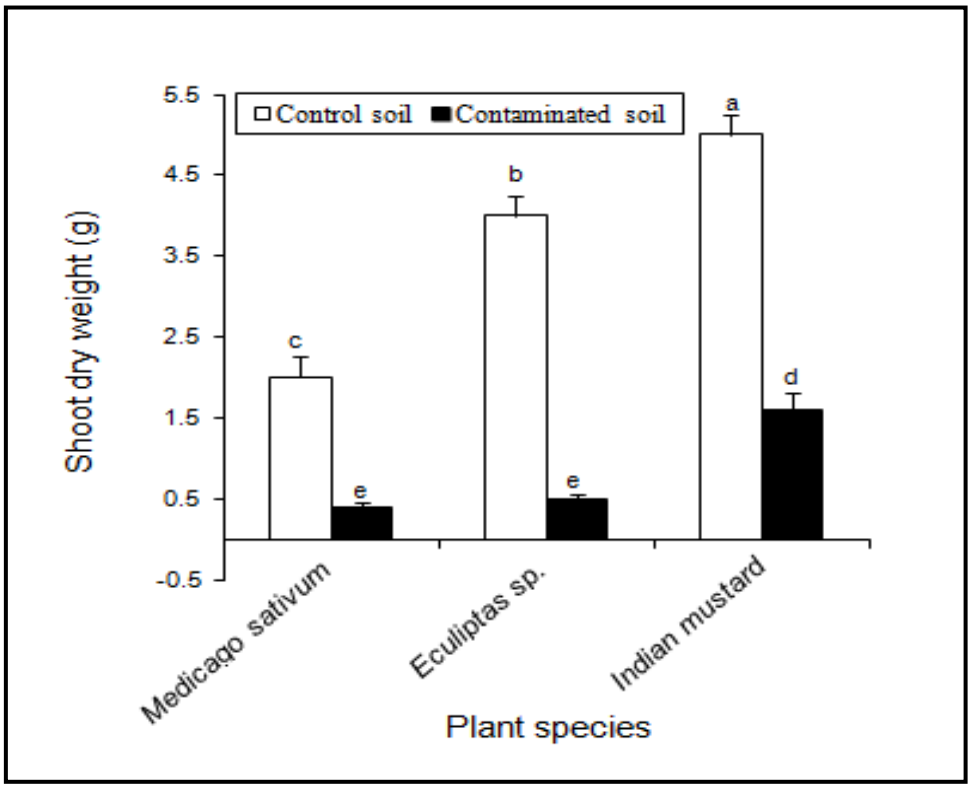

Figure 4.4. Dry hoot weight of plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

4.4.5. The Effectiveness of Plant Species in Pb Uptake by Roots

Figure 4.5 shows the effectiveness of *Eucalyptus camadulensis*, *Indian mustard* and *M. Sativum* seedling on accumulation of Pb in their root. The two–way analysis of variance has revealed that both plant species and soil contamination parameters have significantly influenced the level of Pb in the root seedling. In addition, there was also a significant interaction between plant species and soil contamination in this experiment.

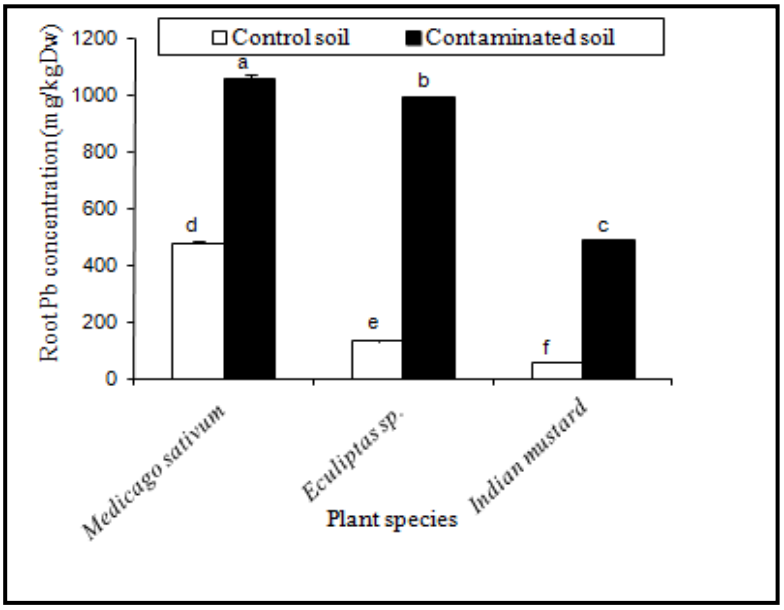

Figure (4.5) Concentration of Pb in root of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

Whether been tested for contaminated or uncontaminated soils, the root of the seedling of *M. Sativum* accumulated greater concentration of Pb than that *for Eucalyptus camadulensis*, which has shown greater values than that for *Indian mustard*. In addition, the level of Pb, accumulated in seedlings grown in contaminated soil was significantly higher than in those grown in uncontaminated ones.

4.4.6. The Effectiveness of Plant Species in Pb Uptake by Shoots

Figure 4.6 represents the effectiveness of *Eucalyptus camadulensis*, *Indian mustard* and *M. Sativum* seedling on accumulation of Pb concentration in their shoots

Similarly, the two–way analysis of variance has also shown that both factors (e.g. plant species and soil contamination) have significantly influenced the level of Pb in the seedlings shoot. In addition, in this experiment, there has also been a significant interaction between plant species and soil contamination.

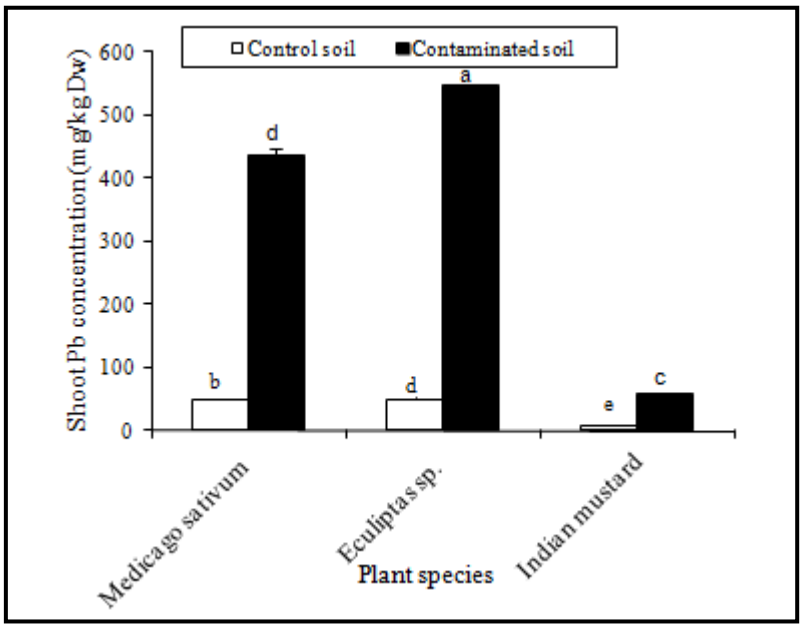

Figure 4.6: Concentration of Pb in shoot of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different P < 0.05. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

In contaminated soils, the level of Pb in the shoot was greater with *Eucalyptus camaldeulensis* than that for *M. Sativum*, which has also shown greater values than that for *Indian mustard*. However, in the uncontaminated soils experiment, the level of Pb in

the plant shoot of *Eucalyptus camaldeulensis* and *M. Sativum* has shown similar values, which were higher than that recorded for *Indian mustard* seedling.

4.4.7. The Effectiveness of Plant Species in Zn Uptake by Roots

Figure 4.7 illustrates the effectiveness of *Eucalyptus camaldeulensis, Indian mustard* and *M. Sativum* on Zn accumulation in their roots. In this experiment, the two–way analysis of variance has indicated that both factors (e.g. plant species and soil contamination) significantly influenced the level of Zn in the root seedlings. In addition, there has also been a significant interaction between plant species and soil contamination.

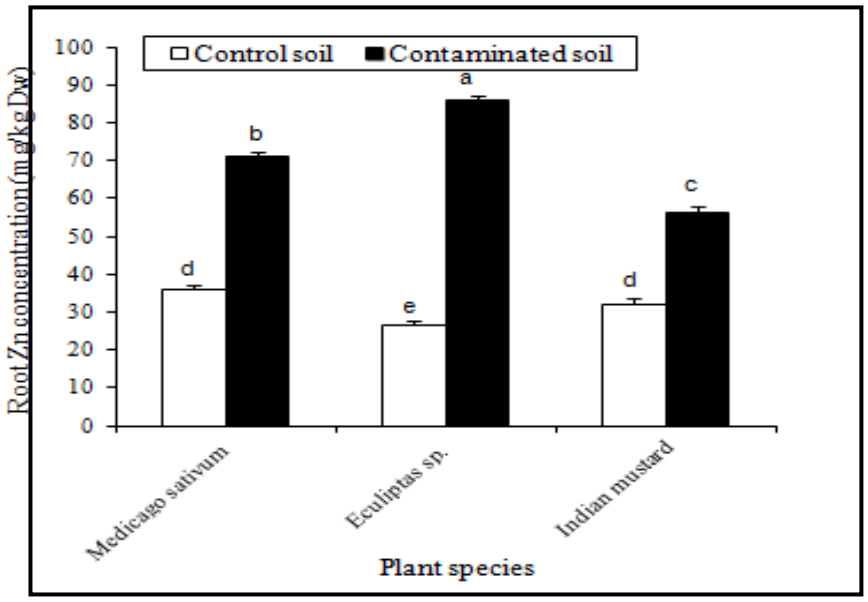

Figure 4.7: Concentration of Zn in roots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different P<0.05. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

In the uncontaminated soils experiment, the obtained results have shown equal amounts of Zn been accumulated in the plant root of *Indian mustard* and *M. Sativum*, which were slightly greater than that observed in the *Eucalyptus camaldeulensis* experiment. However, in the contaminated soils experment, the level of Zn accumulated in the roots of *Eucalyptus camaldeulensis* has shown greater values compared with *M. Sativum*, which has also shown greater values than that for *Indian mustard*. In addition, the results have also shown that levels of Zn were significantly higher in seedlings grown in contaminated soils than those been grown in uncontaminated ones.

4.4.8. The Effectiveness of Plant Species in Zn Uptake by Shoots.

Figure 4.8 shows the effectiveness of Eucalyptus camaldeulensis, Indian mustard and M. Sativum on accumulation of Zn in their shoot. When using two–way analysis of variance, the obtained results have indicated that both parameters (e.g. plant species and soil contamination) have significantly influenced the level of Zn in the seedlings shoot. In addition, this experiment has also shown a significant interaction between plant species and soil contamination.

Likewise, in the contaminated soils experiment, the obtained results have shown that the level of Zn accumulated in the seedlings shoot was more significantly affected in *Eucalyptus camaldeulensis* and *M. Sativum* than that for *Indian mustard*. However, in uncontaminated soils the level of Zn in the shoot of *M. Sativum* seedling was greater than with *Indian mustard*, which has shown greater values than that for *Eucalyptus*

camaldeulensis. In addition, the level of Zn was significantly affected in seedlings grown in contaminated soils than those grown in uncontaminated conditions.

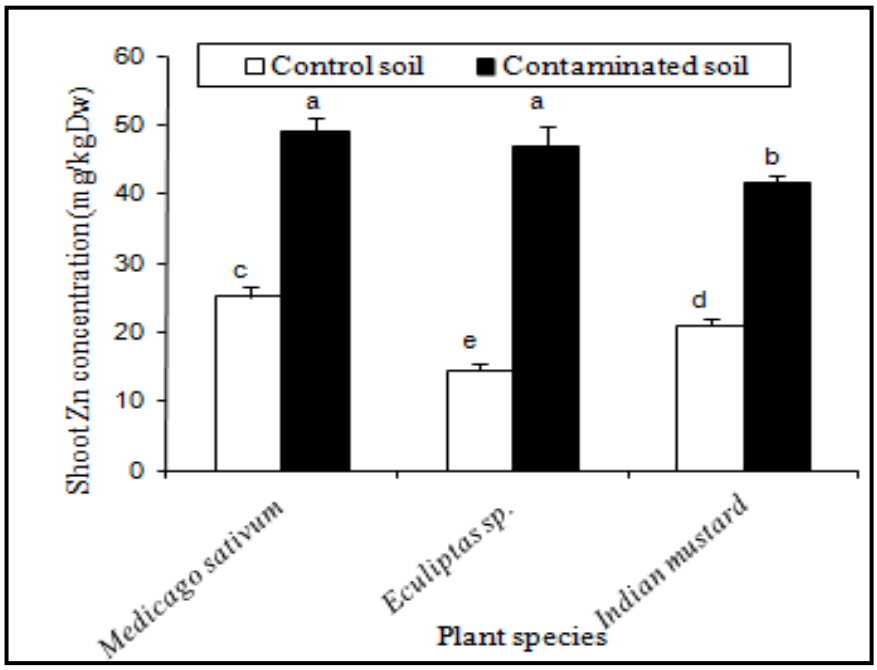

Figure 4.8: Concentration of Zn in shoots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

4.4.9. The Effectiveness of Plant Species in Cu Uptake by Roots

Figure 4.9 presents the effectiveness of plant *Eucalyptus camaldeulensis*, *Indian mustard* and *M. Sativum* on the accumulation of Cu in their root. Two–way analysis of variance indicated that both plant species and soil contamination significantly influenced

the level of Cu in the seedlings root. In addition, there was also a significant interaction between plant species and soil contamination.

In uncontaminated soils, the seedlings of *Eucalyptus camaldeulensis* have shown a greater capacity in accumulating of Cu than was shown by other plant species (e.g. *Indian mustard* and *M. Sativum*). Whereas in this experiment, *Indian mustard and Medicago* have shown equal capacities of accumulating Cu in their seedlings. In regard to the contaminated soils experiment, the obtained results have not shown varied values in the level of the accumulated Cu among plant species.

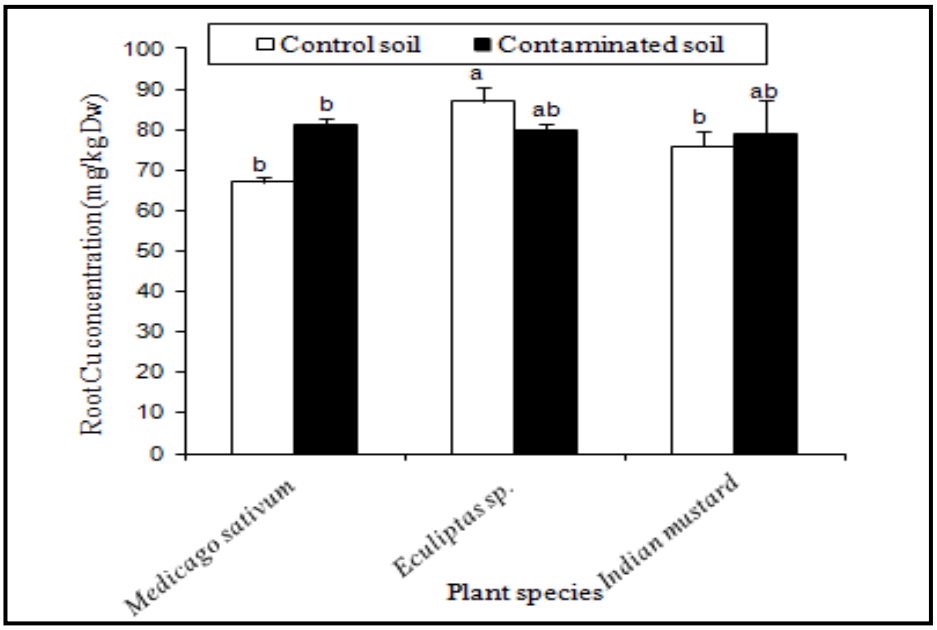

Figure 4.9: Concentration of Cu in roots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different P<0.05. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

4.4.10. The Effectiveness of Plant Species in Cu Uptake by Shoots

Figure 4.10 shows the effectiveness of *Eucalyptus camaldeulensis*, *Indian mustard* and *M. Sativum* on Cu accumulation in their shoot. Two-way analysis of variance indicated that both plant species and soil contamination significantly influenced the level of Cu in the seedlings shoot. In addition, there was also a significant interaction between plant species and soil contamination.

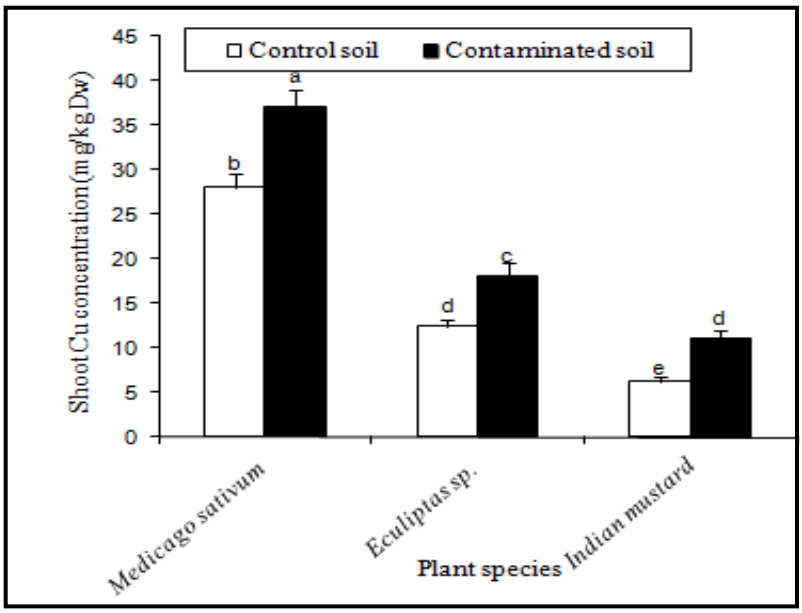

Figure 4.10. Concentration of Cu in shoots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different P<0.05. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

Whether the soils were uncontaminated or contaminated, the level of accumulated Cu was greater with *Medicago sativum* seedling than that for *Eucalyptus camaldeulensis*,

which was greater than with *Indian mustard* seedling. In addition, the level of Cu was significantly higher in seedlings grown in contaminated soil than those grown in uncontaminated ones.

4.4.11. The Effectiveness of Plant Species in Cr Uptake by Roots.

Figure 4.11 shows the effectiveness of *Eucalyptus camaldeulensis, Indian mustard and M. Sativum* on Cr concentration in their root. Two–way analysis of variance indicates that both plant species and soil contamination significantly influenced the level of Cr in the seedlings root. In addition, there was also a significant interaction between plant species and soil contamination.

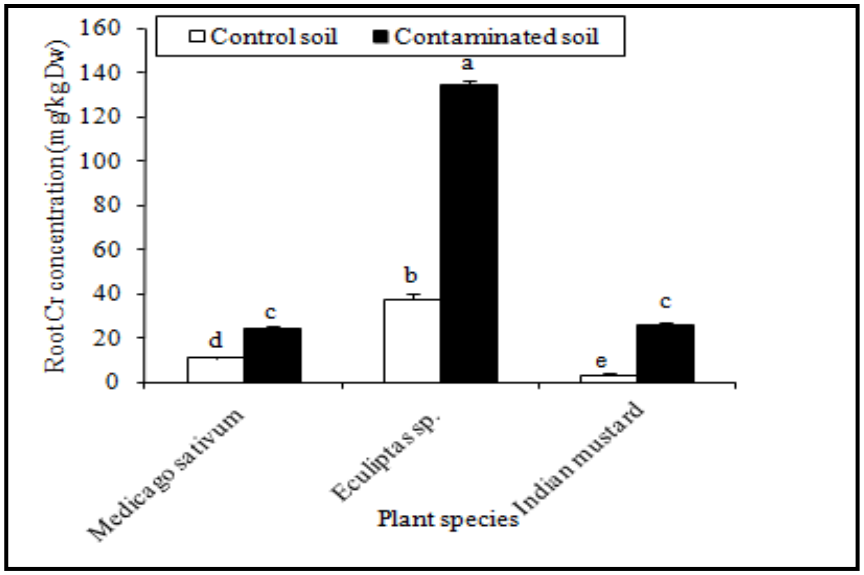

Figure 4.11: Concentration of Cr in shoots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

In uncontaminated soils experiment, the level of Cr accumulate in the plant root of *Eucalyptus camaldeulensis* was greater than with M. *Sativum* seedling and was greater than that for and *Indian mustard* seedling. Moreover, when using contaminated soils, the level of Cr concentration was greater with *Eucalyptus camaldeulensis* than that for both *M. Sativum* and Indian mustard seedling. However, both *Indian mustard* and *Medicago Sativum* accumulated a similar level of Cr. In addition, the level of Cr was significantly higher in seedlings grown in contaminated soil than those grown in uncontaminated ones.

4.4.12. The Effectiveness of Plant Species in Cr Uptake by shoots.

Figure 4.12 shows the effectiveness of *Eucalyptus camaldeulensis*, *Indian mustard* and *M. Sativum* on Cr concentration in their shoot. Two–way analysis of variance indicated that both parameters (e.g. plant species and soil contamination) significantly influenced the level of Cr in the seedlings shoot. In addition, there was also a significant interaction between plant species and soil contamination.

In both experiments conducted on either contaminated or uncontaminated soils, the level of Cr was greater with *Eucalyptus camadulensis* than that for *M. Sativum*, which exhibited greater effect than with *Indian mustard*. In addition, the level of Cr was significantly higher in seedlings grown in contaminated soil than those grown in uncontaminated ones.

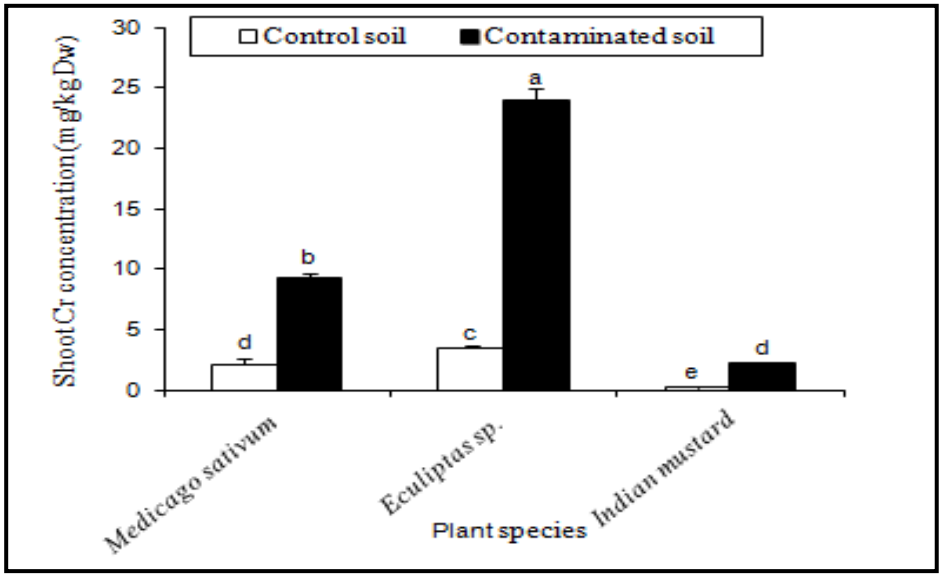

Figure 4.12. Concentration of Cr in shoots and of different plant species grown in a greenhouse using soil collected from two different sites. (Mean values marked with the same letter are not significantly different $P < 0.05$. *Indian mustard =Brassica juncea). a , b ,c , d ,e mean don't share the same letter are significantly different.*

In Table 4.1, the results of P –value for all the green house experiments are summarised.

Table 4.1: Summary of P - value for green houses experiment

	FPR	FPS	DRW	DSW	Pb Root	Pb Shoot	Zn Root	Zn shoot	Cu Root	Cu Shoot	Cr Root	Cr Shoot
Plant spices	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
Soil contamination	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.827**	**<0.01**	**<0.01**	**<0.01**
PS * SC	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.273**	**<0.01**	**<0.01**	**<0.01**

PS = plant spices, SC = Soil contamination, DRW = Dry root weight, DSW = dry shoot weight, FPR = fresh plant root, FPS= fresh plant shoot.

4.4.13. Accumulation and Translocation of Metals in Plants

Table 4.2 shows the accumulation and translocation of Pb, by relation Zn, Cu and Cr in plant species grown in metal contaminated soil, where the BCF is calculated, as the ratio of metal concentration in the roots to that in soil ([Metal]Root/ [Metal]Soil). TF is given as the ratio of metal concentration in the shoots to the concentration of metal in the roots ([Metal] Shoot/[Metal]Root). Whist EF is calculated by as the ratio of the concentration of metal in the shoots to the concentration of metal in the soil (Yoon et al., 2006 and Sun *et al*., 2011).

Table 4.2. Accumulation and translocation of Pb, Zn, Cu and Cr in plant species grown in metal contaminated soil

Plant species	Bio-concentration factor (BCF)				Translocation factor (TF)				Enrichment factor (EF)			
	Pb	Zn	Cu	Cr	Pb	Zn	Cu	Cr	Pb	Zn	Cu	Cr
Medicago sativum	1.4	1	2.7	2	0.9	1.3	0.4	0.4	0.5	0.7	1.1	0.8
Eucalyptus c.	1.1	1.2	3	11	4.1	1.7	0.2	0.1	0.6	0.7	0.7	2.1
Indian mustard	0.5	0.8	2.8	2.2	0.9	1.3	0.1	0.08	0.1	0.6	0.1	0.2

4.5. Discussion

4.5.1. Physico-chemical Properties of Soil

The two soil samples used in this experiment were being conditioned for different physicochemical properties and patterns of pollution (Table 3.1, Chapter 3). Total metal content is an important parameter because it determines the size of the metal pool in the soil, and thus the potential for metal uptake (Ibekwe *et al*., 1995). Therefore, soil samples were analysed for total and extractable metals. Each soil sample exhibited high concentrations in one or more of the metals in question. Soil samples, collected from site 1 (3 m south of the road), were the most contaminated among the collected samples; containing high metal concentrations as shown in Table 3.4. The soil samples collected from this site were highly contaminated with Pb (840 mg kg^{-1}), and been used as contaminated soils compared with control soils collected from Site 15 (10 m south of the road).

4.5.2. Effect of Pb Concentration in Soil on Plant Biomass

Plant species (*Indian mustard, Medicago sativum, and Eucalyptus camadulensis*) were chosen for this study based on their high biomass, and ability to remove heavy metals from contaminated sites (Beladi *et al*., 2011; Turan and Esringu, 2007). All of the three plant species have shown healthy conditions when been grown in the low (control) and high Pb contaminated soils (Figure 4.1). In this experiment, there were higher

differences between the three plant species in shoot and dry root weights (Figures 4.3 and 4.4).

The dry root weights have shown reduced values in all plants grown on Pb contaminated soils compared with similar plants grown on control soils. The dry root weight in all of the three plant species (e.g. *M. sativum*, *I. mustard*, and *Eucalyptus camadulensis*), grown in Pb contaminated soils, were reduced by 4, 3, and 2.5 fold, respectively, lower than that for the dry root weight of similar samples grown on the control soil. The dry shoot weights of *Eucalyptus camadulensis*, *M. sativum*, and *I. Mustard*, grown on Pb contaminated soils, were also reduced by 8, 5, and 3-fold, respectively, compared to similar samples grown in the control soil (Figure 4.5). Similarly, the shoot and root fresh weights were also reduced by 3.4, 4, and 3.8-fold and 2.6, 2.8, and 1.8- fold, in *I. mustard, M. sativum, Eucalyptus camadulensis* and *I. mustard*, respectively, for plants grown in Pb contaminated soil compared with similar plant species grown in control soils (Ali, Khan and Sajad 2013; Pandey 2012) (see also Figure 4.6).

This finding totally agrees with results reported by Turan and Esringu (2007), which is the total of dry weight of *Indian mustard* and *canola* was affected by the contaminated soil; on average, the metals caused a reduction of about 75% in root and shoot dry matters of both plants. Similar results in other hyperaccumulator plants were also reported by other researchers (Ali, Khan and Sajad 2013; Pandey 2012; Chen and Cutright, 2001; Hajiboland, 2005; and Tlustos *et al*., 2006).

4.5.3. Heavy Metal Concentration in Plant Tissues

Metal concentrations in plant tissues also differed among the three plant species grown on similar conditioned soils, indicating their different capacities for metal uptake. Lead concentrations in the roots of plants were comparatively high and varied from 55.8 to 1058 mg kg^{-1} (DW). The highest concentrations of Pb (548 mg kg^{-1}) were detected in the shoots of *Eucalyptus camadulensis* grown in the Pb contaminated soil. Lead concentrations in the root and shoot of *Eucalyptus camadulensis* grown in contaminated soil was about 11 and 7.5 fold, respectively, higher than Pb concentration detected in the root and shoot of similar plants grown on the control soils. However, the Pb concentration in the roots of *M. sativum*, *Eucalyptus camadulensis*, and I. mustard, grown in the Pb contaminated soil was 2.2, 7.5, and 8.6-fold higher than Pb concentrations in the roots of similar plants species grown on control soils (Rajkumar, et al. 2012; Kumar, et al 2013).

Whilst, the Pb concentration detected in the shoots of *M. sativum*, *Eucalyptus camadulensis*, and *I. mustard grown* on the Pb contaminated soil was 8.7, 11.0, and 8.8 - fold higher than Pb concentration in similar plant species grown on control soils. The results indicated that the highest Pb concentration (1058 and 548 mg/ kg-1) was found in the root of *M. sativum* and shoot of *Eucalyptus camadulensis*, respectively, grown in Pb contaminated soil (Figures 4.5 and 4.6). *M. sativum* and *Eucalyptus camadulensis* comparatively accumulated higher Zn concentrations in roots (e.g. 71 and 86 mg kg^{-1}) and shoots (e.g. 49 and 47 mg kg^{-1}), respectively, compared with Indian mustard grown

in similar conditioned soils (Ali, Khan and Sajad 2013; Rajkumar, et al. 2012; Kumar, et al 2013; Pandey 2012).

Similarly, Zn concentrations in the roots of *M. sativum*, *Eucalyptus camadulensis*, and *Indian mustard* were comparatively higher than was detected in the shoots by a factor of 1.4, 1.8, and 1.3-fold, respectively (Figures 4.7 and 4.8). Correspondingly, as shown in Figures 4.9 and 4.10, the highest Cu concentrations (e.g. 81 and 37 mg/kg, DW) were detected in roots and shoots of *M. sativum* grown on Pb contaminated soils. Whereas, the highest Cr concentration (e.g. 133.9 mg/kg, DW) was determined in the roots of *Eucalyptus camadulensis* grown on contaminated soil (Ali, Khan and Sajad 2013; Pandey 2012) (Figures 4.11 and 4.12).

4.5.4. Accumulation and Translocation of Metals in Plants

The mobility of the heavy metals from the polluted substrate into the roots of the plants and the ability to translocate the metals from roots to the harvestable aerial part were evaluated by means of the bioconcentration factor (BCF) and the translocation factor (TF), respectively. BCF is defined as the ratio of metal concentration in the roots to that in soil ([Metal] Root/ [Metal] Soil), whereas, TF is the ratio of metal concentration in the shoots to the roots ([Metal] Shoot/ [Metal] Root). The ability of plants to tolerate and accumulate heavy metals is useful for phytoextraction and phytostabilisation purpose (Yoon *et al*., 2006: and Sun *et al*., 2011). Plants with both bioconcentration factors and translocation factors greater than one (e.g. TF and BCF > 1) have the potential to be used

in phytoextraction. Moreover, plants with bioconcentration factor greater than one and translocation factor less than one (e.g. BCF > 1 and TF < 1) have the potential for phytostabilisation (Yoon *et al*., 2006).

The process of phytoextraction generally requires the translocation of heavy metals to the easily harvestable plant parts, i.e., shoots (Yoon *et al*., 2006), while phytostabilisation process requires a high ability to reduce metal translocation from roots to shoots (Deng *et al*., 2004). By comparing BCF and TF, the ability of different plants in taking up metals from soils and translocating them into the shoots can be compared (Yoon *et al*., 2006). As shown in Table 4.2, among the sampled plants, *Eucalyptus camadulensis* was the most suitable for phytoextraction of Pb and Zn, *whilst M. sativum* and *I. mustard* were more suitable for phytostabilisation of Cu and Cr (Pandey (2012; Rajkumar, et al. 2012)

4.6. Conclusion.

It can be concluded that the three plants used in phytoremediation showed healthy conditions when they were grown in low and high Pb contaminated soil. Howerver, there have been some differences between all of them in terms of shoot and root dry weights compared with the same plants grown on control soils (Figures 4.3 and 4.4). The dry root weight in all of the three plant species (*E. camaldeulensis, Brassica Juncea and Medicago sativum)* were reduced by 4, 3, and 2.5 fold, respectively, lower than the dry root weight of similar samples grown in the control soil. Whilst the dry shoot weights of

the three plants grown in Pb contaminated soils were reduced by 8, 5, and 3-fold, respectively, compared with the similar samples grown in the control soil (Figure 4.5).

Similarly, the fresh shoot and root weights were also reduced in Pb contaminated soil compared with similar plant species grown in control soils. Lead concentrations in the root and shoot of the three plants grown in contaminated soil was higher than Pb concentration detected in the root and shoot of similar plants grown on the control soils. Likewise, Zn, Cu and Cr concentrations in the three plants roots and shoots were higher than the same plants grown in control soils.

From what have been presented above, the TF and BCF for the three plants in this experimental suggests that *E. camadulensis* was suitable for phytoextraction of Pb and Zn, while *M. sativum* and *B. juncea* were suitable for phytostabilisation of Cu and Cr. Furthermore, the findings provided important insight into the feasibility of using, *Eucalyptus camadulensis* in a phytoextraction of lead and zinc role in combination with its growth as a biomass feedstock. *Eucalyptus camadulensis* successfully translocated lead and zinc into harvestable tissues while *M. sativum* and *I. mustard* have successfully shown promising results for phytostabilisation of Cu and Cr.

Next chapter will present the enchancement of phtyremediation using some amendments and *Eucalyptus*, which was the suitable plant for uptaken lead concentration from contaminated soil.

Chapter Five

Enhancement of phytoremediation

The aim of this chapter is to present one of the the present study objectives, i.e. 1.4, which is the assessment of the effectiveness of *Eucalyptus* to absorb and accumulate heavy metal (Pb) in the shoots from Pb contaminated soils. It also attends to evaluate the possible effect of EDTA, *Alcaligenes eutrophos*, compost and Hoagland's solution on the soil and plant. Firstly, a brief introduction of enhancement of phytoremediation will be presented. Secondly, materials and methods applied in this process will be illustrated, which include bacterial inoculation and laboratory trial, and Pot experiment and soil amendments. Then, the findings of the pot experiment are provided. Finally, a discussion of the findings are presented and a conclusions are drawn.

5.1. Introduction

Heavy metal contamination in soils is one of the environmental problems, posing significant risks to human health as well as to ecosystems section 4.1. Therefore, the development of a remediation strategy for metal-contaminated soils is necessary for environmental conservation and human health. Phytoremediation; using plants to remove metal pollutants from contaminated soils, is being developed as a new method for the remediation of contaminated land. Such environmentally friendly, cost-effective and plant-based technology can be economic, aesthetic, and technical advantages over

traditional engineering techniques currently used (Chaney, 1983: Baker *et al*., 1994: Glass, 2000: Susarla *et al*., 2002: and Chaney *et al*., 2005).

Heavy metals in soils are generally bound to organic and inorganic soil constituents, or alternatively, present as insoluble precipitates. A large proportion of metal contaminants are unavailable for root uptake by field grown plants. Methods of increasing heavy metal contaminant phytoavailability in soil and its transport to plant roots are vital to the success of phytoremediation in the field (Ernst 1996: and Kukier *et al*., 2004).

An amendment can be a physical, chemical, natural or synthesised compound, which improves the physio-chemical properties of soil against unwanted event/s, such as; contamination, wind erosion or as a tool for solving environmental soil problems, sediments, water and air (Black *et al*., 1993; De-Souza *et al*., 1999). Amendments may be added in order to enhance phytoextraction of heavy metals (mobilisation), or to stabilise the soil in order to prevent leaching of heavy metals to the ground water and allow plant growth in polluted sites (Wu *et al*., 2004). Mobilisation, in situ, chemically enhances soil flushing by extracting solutions, such as organic and inorganic acids, and complexation agents, which is an example of a method used for remediation (Grcman *et al*., 2001: Vulava and Seaman, 2000). For example, EDTA enhanced the phytoextraction of Pb and Zn more than salts $(NH_4)2SO_4$ and NH_4NO_3 (Zhuang *et al*., 2005). At high concentrations, EDTA affects plants growth (Meers *et al*., 2005a) and enhances the

mobility of Cu and Pb in the soil, but not Zn and Cd (Black *et al*., 1993; De-Souza *et al*., 1999: Wu *et al*., 2004).

It was also discovered that as amendments were added, there was a recorded increase in plant biomass. Biomass increase occurred as follows: compost > topsoil > urea, it was also reported (Solhi *et al*. 2005; Wenzel *et al*., 2003) that the amendments added improved the soil's chemical and physical properties. The mobilisation of amendments depends on the type of amendment added and the variety or species of plant. For example, in a research study conducted by Meers *et al.* (2005), it was found that the amendments; EDTA or DTPA, does not affect the phytoextraction by canola plants, but rather increases the liability of heavy metals to leach to ground water. In a similar study, Solhi *et al*. (2005) investigated the effect of three amendments (manure, sulphuric acid and DTPA) on two crops, sunflower (*Helianthus annuus*) and canola (*Brassica napus*). It was indicated that the manure gave higher biomass production and the sunflower had a higher extracting potential for Pb and Zn removal from polluted soil.

Microbial populations are known to affect trace metal mobility and availability to the plant, through the release of chelators, acidification, and redox changes (Smith and Read, 1997; Abou-Shanab *et al*. 2003b). The presence of rhizosphere bacteria has been reported to increase the concentrations of Zn, Cu, Pb or Cr in plants (Whiting *et al*., 2001; Chen *et al*., 2005). When interactions between plants and beneficial rhizosphere microorganisms are improved, it enhances biomass production and tolerance of plants to heavy metals; this is considered to be an important component of phytoremediation

technologies (Wenzel and Jockwer, 1999; Glick, 2003). Therefore, the use of microorganisms in combination with plants is expected to provide high efficiency for phytoremediation (Whiting *et al*., 2001). For example, chemolithotrophic bacteria have been shown to enhance the environmental mobility of metal contaminants via soil acidification, or in contrast, to decrease the solubility due to precipitation as sulphides (Abou-Shanab *et al*. 2003a; Guan *et al*., 2001). Studies have shown that microbes influence root parameters, such as; root morphology and growth. An increase in root exudation of organic solutes could affect the rate of phytosiderophore release. In turn, rhizosphere microoganisms may interact symbiotically with roots to enhance the potential for metal uptake (Nautiyal, 1999; Burd *et al*., 2000; Guan *et al*., 2001).

The use of hyperaccumulator species in continuous phytoextraction processes is limited by the low bioavailability of these pollutants in root uptake (Salt *et al*., 1998; Peer *et al*., 2005). On the other hand, the majority of hyperaccumulators present a slow growth rate leading to a low annual biomass (Peer *et al*., 2005). In the last ten years, it was demonstrated that the application of mobilising/chelating agents, such as ethylene diaminetetraacetic acid (EDTA), to soil is a reliable practice to increase plant metal bioavailability, uptake and shoot accumulation (Evangelou *et al*., 2007; Meers *et al*., 2008; Luo *et al*., 2008). Such chelators have a strong affinity for different heavy metals cations (Cooper *et al*., 1999; Romkens *et al*., 2002; Shen *et al*., 2002; Meers *et al*., 2005) and are more readily translocated from the roots to the shoots as a metal–chelate complex (Lombi *et al*., 2001; Collins *et al*., 2002; Wenger *et al*., 2003; Tandy *et al*., 2006). Hence,

chelators can simultaneously enhance the uptake and translocation of heavy metals (Wu *et al*.,2004; Tandy *et al*., 2006), and can also reduce the toxicity of free metal-cations in the photosynthetic organs by complexation (Vassil *et al*., 1998; Greman *et al*., 2003; Hernandez-Allica *et al*., 2003). Chemically induced phytoextraction plants become fast growing, have the capability to tolerate and accumulate high concentrations of metal in their shoots amongst many other characteristics (McGrath *et al*., 2002; Shen *et al*., 2002; Van Engelen *et al*., 2007; Quartacci *et al*., 2006).

The addition of fertiliser and compost are the most common methods for increasing crop productivity, and can also be used to increase plant biomass. In China, chicken manure and urea are among the main fertilisers used in agricultural production (You, 1995; Yang, 2002). Chicken manure and urea were added to soil to explore the effect on cadmium (Cd) accumulation by *Solanum nigrum L;* a newly found Cd-hyperaccumulator with high phytoextraction efficiency (Wei *et al*., 2005). *S. nigrum* is a weed, that once fertiliser is added, its biomass can grow rapidly; therefore, it is a better material to use to determine the effects of fertilisers on phytoextraction (Wei *et al*., 2005). Some bacteria, such as the heavy metal resistant *Alcaligenes eutrophos CH34* strains, are able to promote biomineralisation; which is biologically induced crystallisation of heavy metals (Mahvi *et .al,* 2004). In the presence of heavy metals, this strain may create an alkaline environment in the periplasmic space and outer cell environment and appropriate induction of heavy metals resistance mechanisms (Mahvi *et .al,* 2004). The use of

Alcaligenes eutrophos in this study is due to its heavy metal resistance at high concentration.

The study reported in this thesis was carried out to assess the effectiveness of *Eucalyptus* to absorb and accumulate heavy metal (Pb) in the shoots from Pb contaminated soils. It also aims to evaluate the possible effect of EDTA, *Alcaligenes eutrophos*, Compost and Hoagland solution application.

5.2. Materials and Methods

5.2.1. Bacterial Inoculation

Alcaligenes eutrophus bacterial cells were grown overnight in 500 ml Elenmeyer flasks containing 250 ml sterilised nutrient broth on a shaker at 150 rev/min at 30ºC until late log phase was reached in two hours. The absorbance was measured every hour for a 24 hr period. Bacterial cells were harvested by centrifugation and the pellets were washed twice with sterile saline. 50 µg of soil samples were added to each dilution from (10^{-1} to 10^{-8} and incubated for 48h at 37°C.

5.2.2. Laboratory Trial.

In the laboratory, 5g portions of soil samples injected with the amendment were weighed in 100 ml glass flasks. The soil samples to be examined where: the compost (0%, 5%, 10%, 25%), EDTA (0 mmol, 5 mmol, 10 mmol, 15 mmol), Hoagland solution (0%, 25%, 50%, 100%), Bacterial inoculum and mixed amendments (5% compost + 5

mmol EDTA + 25% Hoagland solution + bacterial inoculum). All experiments were replicated 3 times. All flasks were kept in the laboratory for two weeks. After two weeks, the Pb availability was determined by ICP–AES. Soil pH, conductivity, total organic content, total nitrogen and phosphorus have been determind. These terms have described in Chapter 3, section 3.1.2.4, 3.1.2.6-7 and 3.1.2.10.

5.2.3. Pot Experiment and Soil Amendments

In practice, approximately 2 kg of air-dried soil was placed into plastic pots (pots were 18 cm in diameter and 13 cm in depth). Seeds of *Eucalyptus* Sp. were sown in plastic pots that contained metal (Pb) contaminated soil (840 mg/kg dry soil) with four replicates for each treatment. The experiment was carried out in a greenhouse illuminated with natural light at 25 ^{o}C. After germination, the seedlings were thinned to two plants per pot and grown for 90 days.

To prepare the bacterial inocula (*Alcaligenes eutrophus*), bacterial cells were grown overnight in 500 ml Erlenmeyer flasks containing 250 ml of sterilised nutrient broth and placed on a shaker at 150 rpm/min at 30^{o}C until late log phase in two hours. Bacterial cells were then harvested by centrifugation (4000 g×, 20^{o}C, 10 min), and the pellets were washed twice with sterile saline solution. Bacterial suspensions in the saline solution were then adjusted to an absorbance of 0.5 at 600 nm (e.g. equivalent to approximately 7.4×10^{8} c.f.u. ml^{-1}), which were used for soil inoculation (Abou-Shanab *et al*. 2003b).

Eucalyptus seeds were sown in plastic pots containing 2 kg Pb contaminated soil from Azzawiyah. After 2 weeks, the plants were reduced to 3 plants per pot. 4 weeks after seed germination occurred, 5 ml of the appropriate bacterial suspension was added at a concentration of 10^8 c.f.u ml^{-1} (sterile distilled water) whereas for the non-inoculated control plants, the same amount of sterile distilled water was added after plant seedlings were spotted (which occurred after 4 weeks). Other pots were amended with three different concentrations of EDTA (ethylenedinitrilotetraacetic acid) i.e. (0, 5, 10, and 15 mmol/kg soil) and control plants were not treated with EDTA.

Other experimental pots contained Pb contaminated soils amended with 5%, 10% and 25% (wt/wt soil: compost). The compost was purchased from agrovista, precision services. The physicochemical properties of the compost are shown in Table 5.1.

Other series of pot experiments were irrigated with different dilutions of Hoagland's solution (25, 50, and 100%) twice a week. Mixed amendments *(5% compost + 5 mmol EDTA + 25% hoagland solution + 5ml bacterial suspension)* were used in other pots. All the pots were put randomly. Plants were grown for three months in a greenhouse illuminated with natural light, which has been mentioned above. At the end of the experiment, the plant will be collected.

Table 5.1. Physical and chemical characteristics of compost (agrovista, precision)

Analysis	Result
pH	7.3
Electrical Cond mmhos/cm	4.10 mg/kg^{-1}
Dry Matter	60.68 mg/kg^{-1}
Total Nitrogen mg/kg	1050.3 mg/kg^{-1}
Phosphorus mg/kg	65.4 mg/kg^{-1}
Potassium	7225.6 mg/kg^{-1}
Magnesium	970 mg/kg^{-1}
Calcium	6950 mg/kg^{-1}
Sodium	1400 mg/kg^{-1}
Lead	20.01 mg/kg^{-1}
Nickel	15.70 mg/kg^{-1}
Cadmium	0.10 mg/kg^{-1}
Chromium	20.11 mg/kg^{-1}
Arsenic	1.80 mg/kg^{-1}

5.2.4. Plant harvesting and metal analysis.

After three months, plants were gently removed from the pots. Shoot and roots were separated and the weights of both were measured. Plant shoots and roots were washed with deionised water, rinsed, and dried at 70ºC, the dry matter (DM) was then measured. Plant materials were grounded and two grams milled plant matter was digested with a mixture of HCl/HNO (4:1, v/v). The Pb element was determined as described in sectioon 3.1.2.10 using ICP-AES analysis.

5.2.5. Bioconcentration and Translocation factor

The bioconcentration factor (BCF) provides an index for the ability of the plant to accumulate metal with respect to the metal concentration in the substrate. The calculation of these factors has been described in Chapter 4, section 4.4.13

5.2.6. Statistical Analyses

Data was analysed using Minitab software (Version 16 English), the variance was tested in significant results between variables (ANOVA) separated using a Tukey test where $p \leq 0.05$.

5.3. Results

5.3.1. Bacatrial Soil Survival

Table 5.2 shows the sterilised and non-sterilised soil experiment results, and indicates that the amount of bacteria in non-sterilised soil with amendments is 1.3×10^5 CFU g^{-1}; whereas sterilised soil with amendments is considerably lower. Results for sterilised soil without any amendments are zero and for non-sterilised soil without bacterial inoculum the result is 2×10^3 CFU g^{-1}.

Table 5.2: The total culturable bacteria isolated from sterilised and non-sterilised soil amended and un-amended with *Alcaligenes eutrophus* bacteria n =3

Non sterilised soil	**Sterilised soil**	**Non sterilised soil**	**Sterilised soil**
Amended with bacterial inoculums		*Without bacterial inoculum*	
CFU g^{-1}			
1.3×10^5	9.5×10^4	2×10^3	0

5.3.2 The Effect of Different Amendments on Available Pb Concentration

Table 5.3 shows that the highest level of extractable metal is associated with EDTA concentration of 15 mmol containing both bacterial inoculums and mixed amendments. Whilst the extractable metal for compost and Hoagland solution have similar results with minimal variations; results showed that the concentration of Pb with no compost was 8.8 mg Pb kg^{-1}, when the level of compost was increased to 5%, Pb concentration increased to 9.1 mg Pb kg^{-1}. Moreover, the concentration reached 9.5 mg Pb kg^{-1} when the level of compost was increased to 10%. However, the concentration of Pb decreased to 7.6 mg Pb kg^{-1} when the level of compost was elevated to 25%.

The obtained results also indicated that the concentration of Pb increased with increasing EDTA concentration. With solutions with no added EDTA, the concentration of Pb was 8.8 mg Pb kg^{-1}, this concentration increased to 12.9 mg Pb kg^{-1} when EDTA concentration was increased to 5 mmol. Furthermore, Pb concentration increased to 14.3 and 17.3 mg Pb kg^{-1} when the concentration of EDTA increased to 10 mmol and 15 mmol, respectively. The addition of Hoagland solution caused an increased concentration of Pb from 8.8 mg Pb kg^{-1} at 0 % to 9.1 mg Pb kg^{-1} at 25% of Hoagland solution; whilst, the Pb concentration remained nearly constant at 10.3 and 10.1 mg Pb kg^{-1} for Hoagland solution concentration of 50% and 100%, respectively. In addition, Pb concentration was 11.6 mg Pb kg^{-1} when bacterial inoculums' was used and 15.1 mg Pb kg^{-1} when mixed amendments were applied.

Table 5.3: Effect of different amendments on available Pb concentration in roadside soil contaminated with Pb[a] n=3

Amendments	**[b]$CaCl_2$ extractable metal (mg Pb kg^{-1} dry soil)**
Composite (C)	
0%	8.8 ± 0.1
5%	9.1 ± 0.3
10%	9.5 ± 0.4
25%	7.6 ± 0.5
EDTA (E)	
0 mmol	8.8 ± 0.1
5 mmol	12.9 ± 0.3
10 mmol	14.3 ± 0.6
15 mmol	17.3 ± 1.5
Hoagland solution (H)	
0%	8.8 ± 0.1
25%	9.1 ± 0.3
50%	10.3 ± 0.2
100%	10.1 ± 0.2
Bacterial inoculum's (B)	11.6 ± 0.6
Mixed amendments ***(5%C+ 5 mmol E+ 25% H+B)***	15.1 ± 0.7

[a] total Pb concentration (840 mg/g dwt); [b] two hours after addition

5.3.3. Physio-chemical characteristics of Pb contaminated soil with different amendment

Table 5.4 indicates that pH increased from 7.7 to 8.2 when compost concentration was increased from 0 to 25%, while with other factors, such as an increase in % EDTA

concentration, Hoagland solution (%), bacterial inoculum and mixed amendments, the pH level decreased.

Table 5.4: Mean physico-chemical characteristic results of Pb unplanted, contaminated soil with different amendments after one month in pots, where n=3.

Amendments	**pH**	**CEC**	**EC**	**OM**	**N**	**P**	**Pb *Available***
			mmhos cm^{-1}	(%)	mg kg^{-1}		mg kg^{-1}
Composite (C)							
0%	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
5%	7.81	8.21	2.12	1.15	0.52	0.75	9.6 ± 0.15
10%	8.10	8.28	2.98	2.35	0.92	0.81	10.2 ± 0.25
25%	8.20	8.33	3.15	4.54	2.31	0.98	12.6 ± 0.47
EDTA (B)							
0 mmol	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
5 mmol	7.68	8.11	2.05	0.09	0.13	0.72	14.2 ± 0.86
10 mmol	7.64	8.15	2.51	0.11	0.13	0.81	16.8 ± 0.30
15 mmol	7.60	8.25	2.98	0.12	0.15	0.83	17.6 ± 0.68
Hoagland solution (H)							
0%	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
25%	6.54	8.25	1.82	0.16	0.19	0.81	9.5 ± 0.20
50%	6.40	8.51	2.34	0.25	0.25	0.93	11.6 ± 0.74
100%	6.05	8.95	2.87	0.37	0.48	1.52	13.1 ± 0.27
Bacterial inoculum's (B)	6.6	8.3	1.68	0.15	0.14	0.90	15.4 ± 0.66
Mixed amendments *(5% C+5 mmol E+ 100% H+B)*	6.9	9.05	2.35	0.20	0.55	2.25	17.2 ± 0.98

In terms of CEC results, Table 5.4 showed that CEC levels increased from 8.09 % to 8.33% when 25% compost in 15 mmol EDTA was added. CEC levels for 100% Hoagland solution increased from 8.25 to 8.95. Moreover, the CEC value was 8.3 with bacterial inoculum and 9.05 with mixed amendments. The same scenario was observed for EC. The EC for the control sample was recorded as 1.6 mmhos cm^{-1}, this increased to 3.15, 2.98 and 2.87 mmhos cm^{-1} when 25% compost, 15 mmol EDTA and 100% Hoagland solution were applied respectively. Furthermore, the EC value was 1.68 mmhos cm^{-1} when Bacterial inoculum was used and 2.35 mmhos cm^{-1} when mixed amendments were applied.

As shown in Table 5.4, the increase in compost level had a positive effect on the amount of organic matter content (OM precentage). The OM% was found to be 0.05% for the control and increased to 4.54% when the amount of compost was increasde to 25%. However, the effect of increasing the concentration of EDTA from 0 to 15mmol was not clear on OM% (e.g. OM% was 0.09 at 0 mmol EDTA and 0.012 at 15mmol EDTA, respectively). On the contrary, as Hoagland solution percentage increased, the OM% also showed a slight increase (e.g. OM% was 0.09 with control samples and then rose to 0.37 for 100% of Hoagland solution). The OM% for bacterial inoculum and mixed amendments was 0.15 and 0.20, respectively.

Table 5.4 showed that the concentration of N and P remarkably increased from 0.14 and 0.70 $mg.kg^{-1}$ which were the N and P results for the control samples, to 2.31 and 0.98 $mg.kg^{-1}$, respectively when 25% of compost was applied. However, increasing

EDTA concentration from 0 to 15 mmol had no clear effect on the concentration of N and P in this experiment. Moreover, an increase in level of Hoagland solution from 0% to 100% increased N concentration from 0.14 to 0.48 $mg.kg^{-1}$ and increased P concentration from 0.70 to 1.52 $mg.kg^{-1}$, respectively. It was also noted that bacterial inoculum had no effect on N concentration; however it did increase P concentration to 0.90 $mg.kg^{-1}$. In addition, when mixed amendments were added to the samples, increases were seen in N and P to to 0.55 and 2.25 $mg.kg^{-1}$, respectively. Moreover, it can be clearly noticed that the highest level of available Pb was with EDTA 15 mmol; which produced 17.6 $mg.kg^{-1}$ available Pb, bacterial inoculum produced 15.4 $mg.kg^{-1}$ available Pb and mixed amendments gave 17.2 $mg.kg^{-1}$. The obtained results also show that the lowest levels of available Pb are associated with the application of compost and Hoagland solution (e.g. 12.6 and 13.1 $mgkg^{-1}$ were levels of available Pb obtained for compost and Hoagland solution, respectively).

5.3.4. The Effect of Soil Contamination with Different Amendment on the Root and Shoot Fresh Weights

Table 5.1 shows the effect of soil amendments on the resh root weight and shoot weight in which one way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicated that the fresh weight of the root of the plant was significantly influenced by soil amendments. The F and P values were 551.2 and < 0.001, respectively. In comparison to the untreated control, all applied amendments (apart from EDTA 5 mmol), enhanced the fresh root

weight. The data shows that the mixed amendment is the most effective treatment followed by Hoagland solution 100% in improving the fresh root weight.

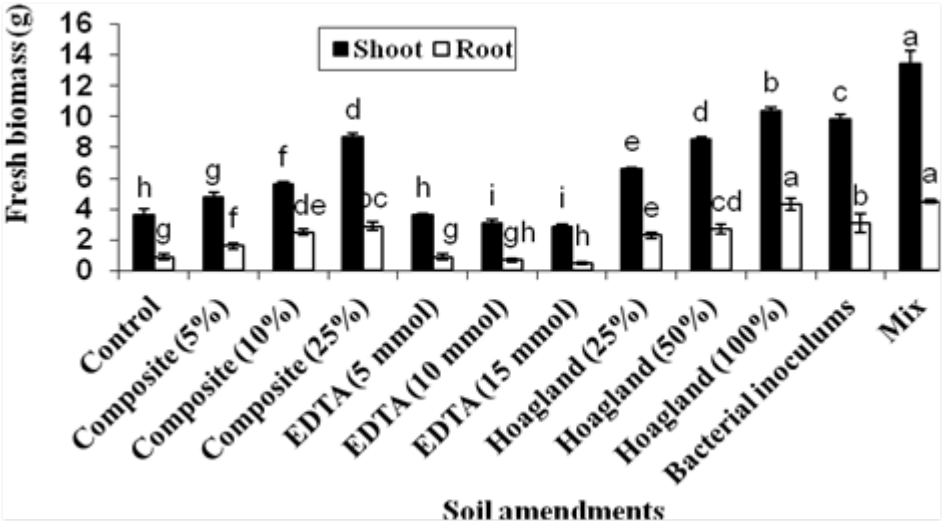

Figure 5.1: Shoot and root fresh weights of plant species grown in a greenhouse. Mean values marked with the same letter are not significantly different $P<0.05.n=3$

The effect of soil amendments on the fresh shoot weight is shown in Figure 5.1. One way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicates that the fresh weight of the plant shoot was statistically influenced by soil amendments. The F and P values were 2894 and < 0.001, respectively. In comparison to the untreated control, all applied amendments (apart from EDTA 5 mmol), enhanced the fresh shoot weight. The data shows that mixed amendment was the most effective treatment followed by Hoagland solution 100% in improving the fresh shoot weight.

5.3.5. The Effect of Soil Contamination with Different Amendment on the Shoot and Root Dry Weights

The effect of soil amendments on the root and shoot dry weights are shown in Figure 5.2.

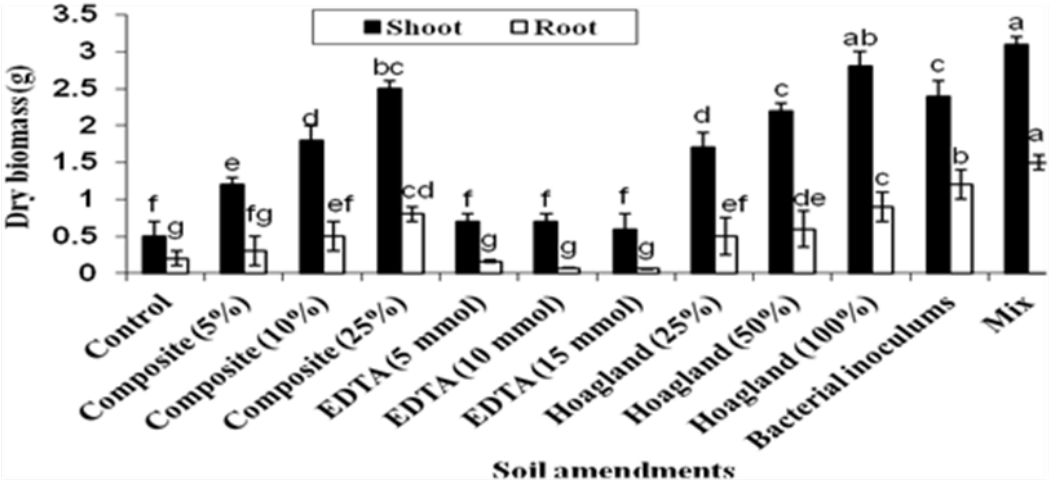

Figure 5.2 Shoot and Root dry weights of plant species grown in a greenhouse. Mean values marked with the same letter are not significantly different P<0.05.n=3

One way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicates that the dry weight of plant roots was significantly influenced by soil amendments. The F and P values were 63.2 and < 0.001, respectively. In comparison to the untreated control, all applied amendments (apart from EDTA 5 mmol and 5% compost), enhanced the dry root weight.

The data revealed that the most effective treatment in increasing the dry root weight was mixed amendment followed by bacteria.

The effect of soil amendments on the dry shoot weight is also shown in Figure 5.2. One way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicates that the dry weight of shoot was significantly influenced by soil amendments. The F and P values were 172 and < 0.001, respectively. In comparison to the untreated control, all applied amendments (apart from EDTA 5 mmol), enhanced the dry shoot weight. The data revealed that the most effective treatment in increasing the dry shoot weight was mixed amendment followed by Hoagland solution 100%.

5.3.6. The Effectiveness Amendments of Plant Species in Pb Uptake by Roots and Shoots.

Figure 5.3 shows the effect of soil amendments on root's Pb concentration. One way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicates that the Pb root concentration was significantly influenced by soil amendments. The F and P values were 570 and < 0.001, respectively. In comparison to the untreated control, all applied amendments improved the root's ability to accumulate Pb. The data revealed that the most effective treatment in improving root's Pb concentration was the addition of bacteria followed by EDTA 15mmol and mixed treatment. Regardless to the treatment method, increasing the level of applied amendments generally improved the root's Pb concentration.

Figure 5.3 also shows the effect of soil amendments on the shoot's Pb concentration. One way analysis of variance at $P \leq 0.05$ (Tukey HSD) indicates that the Pb shoot concentration was significantly influenced by soil amendments. The F and P values were 9.7 and < 0.001, respectively. Bacteria and ETDA 15mmol were the only treatments that increased the level of Pb in the shoot greater than the untreated control samples. Although ETDA 10mmol accumulated similar levels to those treated with bacteria and ETDA 15mmol, the value was similar to the rest of the treatments.

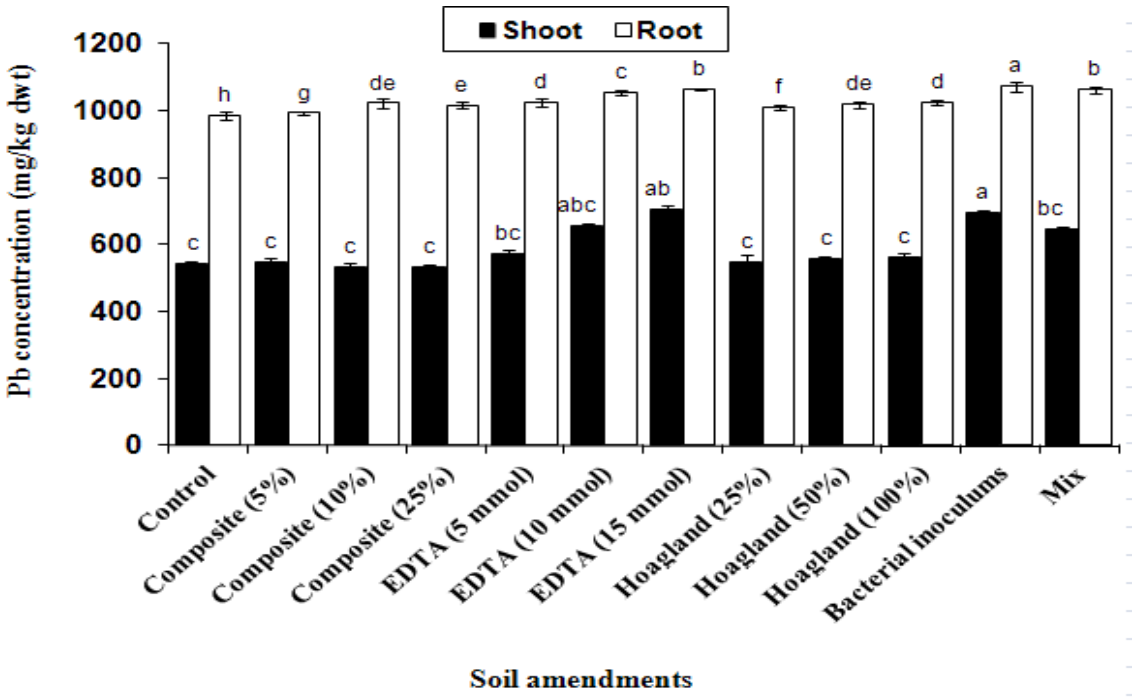

Figure 5.3: Concentration of Pb in shoot and root of plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly different P<0.05.n=3

5.3.7. Accumulation and translocation of Pb in plant grown in Pb contaminated soil mixed with different amendments

The accumulation and translocation of Pb grown in lead contaminated soil mixed with different amendments are shown in Table 5.5.

Table 5.5: Accumulation and translocation of Pb in plants grown in Pb contaminated soil mixed with different amendments

Amendment	Bio-concentration factor (BCF)	Translocation factor (TF)
Control	1.17	0.55
Composite (5%)	1.18	0.55
Composite (10%)	1.03	0.52
Composite (25%)	1.21	0.52
EDTA (5 mmol)	1.22	0.56
EDTA (10 mmol)	1.25	0.62
EDTA (15 mmol)	1.26	0.66
Hoagland (25%)	1.2	0.54
Hoagland (50%)	1.21	0.54
Hoagland (100%)	1.22	0.55
Bacterial inoculums	1.27	0.64
Mixed amendments	1.26	0.6

*BCF is calculated by relation: Ratio of metal concentration in the roots to that in soil ([Metal]Root/ [Metal]Soil), TF is given by relation: The ratio of metal concentration in the shoots to the concentration of metal in the roots ([Metal]Shoot/[Metal]Root) (Yoon *et al*., 2006; Sun *et al* ., 2011).

5.4. Discussion

5.4.1. Influence of Different Soil Amendments on Pb Availability in Soil

The bulk of soil metals are insoluble compounds that cannot be transported, consequently, this affects the metal uptake of hyper-accumulating plants. Recently, many synthetic chelators, such as EDTA (ethylenediaminetetraacetic acid), and EDDS (S, S-ethylenediaminedisuccinicacid) have been applied in Pb-contaminated soils to increase the mobility and bioavailability of Pb, thereby increasing the amount of accumulated Pb in the shoots of phytoextracting plants (Luo *et al*., 2006; Turgut *et al*., 2005). Among these chelators, EDTA has been found to be the most efficient in increasing the concentration of water-soluble Pb (Wu *et al*., 1999; Kumar *et al*., 2011). Stabilisation of inorganic contaminants by the processes of adsorption, binding or co-precipitation with the additive amendments (Kumpiene *et al*., 2008), have been widely researched in the current decade (Madejon *et al*., 2008). Of the numerous amendments used for in situ stabilisation of contaminants, organic materials such as biosolids, manures and composts, rich in organic matter, have proved successful at reducing the mobility of contaminants in multi-metal polluted soils (Mench *et al*., 2010; Alvarenga *et al*., 2008). Soil microorganisms can affect trace metal mobility and availability to the plant; they can produce iron chelators and siderophore for ensuring iron availability, reducing soil pH, and/ or solubilising metal-phosphates. As shown in Tables 5.2 and 5.3, the addition of EDTA, bacterial inoculum, and a mixture of EDTA, nutrient solution and compost all led to an increase in the availability of Pb in soil. The total nitrogen and phosphorus content

were increased as a result of the addition of Hoagland solution and compost as shown in Table 5.3. The addition of compost has led to a slight increase in the available Pb concentrations; as a result of the total Pb concentration in compost as shown in table 5.1.

5.4.2. The Effect of Soil Amendments on the Plant Biomass

The effect of soil amendments on the biomass of *Eucalyptus Sp*, in terms of its fresh and dry weight, is presented in Figure 5.1. The effect of applying EDTA on the biomass of Eucalyptus plants showed a significant decrease in biomass (both in fresh and dry weight) at the highest concentration (15 $mmol.kg^{-1}$ EDTA) (refer to Figure 5.1 for more information). However, there were no significant effects when lower concentrations of EDTA were applied in comparison to the control samples. The fresh shoot weight in Eucalyptus plants grown in Pb contaminated soils amended with EDTA (10 and 15 $mmol.kg^{-1}$ soil) were reduced 1.1 and 1.2 times lower than that for the fresh shoot weight of the same plants grown in the control soil, respectively. The fresh shoot weight of Eucalyptus plants grown in Pb-contaminated soil amended with compost (5%, 10%, and 15%), Hoagland solution (25%, 50%, and 100%), bacterial inoculums, and mixture of previous amendments plus EDTA (5%) were increased by 1.2, 1.8, 2.5, 1.7, 2.2, 2.8, 2.4, and 3.1 fold, respectively in comparison to the control samples (refer to Figures 5.1 and 5.2).

5.4.3. Effect of Soil Amendments on the plants ability to accumulate Pb

The application of EDTA (5%, 10% and 15%) increased Pb accumulation in the shoot by 1, 1.2, and 1.3 times in comparison to the control samples (refer to Figure 5.3).

The application of EDTA promoted Pb accumulation significantly in B. juncea when grown in artificially contaminated soil (Kumar *et al*., 2011). The accumulation of Pb in shoots increased further with increasing EDTA concentration, than that in roots. The highest accumulation of Pb (371.433 $mg.kg^{-1}$ DW) was noticed with the highest dose of EDTA (10 mmol EDTA kg^{-1}) and Pb (500 mg Pb kg^{-1}) in shoots (Kumar *et al.*, 2011). Bacterial inoculation led to a noticeable increase in the Pb accumulation in the shoot by 1.3 times higher than the control samples (Figure 5.3). Rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Burd *et al*., 2000; Guan *et al*., 2001). Abou-Shanab *et al*. (2003b) reported that the concentration of extractable Ni increased to 2.2 to 2.6 $mg.kg^{-1}$ when the applied soil samples were inoculated with *M. arabinogalactanolyticum* AY509224.

The results obtained from this study clearly demonstrate that some soil amendments can rapidly and dramatically reduce the time required for remediation with increased Pb concentrations in soil solution. It also reduces the time required for Pb translocation from roots to shoots in the chosen plants (Piechalak *et al*., 2003; Hovsepyan *et al*., 2005; Lai *et al.*, 2006). Similar studies also found that EDTA is probably the most studied amendment (conditioning additive) in phytoremediation research (Piechalak *et al*., 2003; Hovsepyan *et al*., 2005). It has successfully been utilised to enhance phytoextraction of lead and other metals from contaminated soils (Zhao *et al*., 2010).

Furthermore, the pot experiments, in this study, certainly confirm that the addition of EDTA has increased bioavailability of Pb in soil, which increases the uptaken of lead

from roots and shoot of *E. camaldulensis.* These results are in agreement with the view that EDTA is the most efficient chelating agent to increase Pb accumulation in plant shoot (Kumar, et al 2013; Blaylock *et al*., 1997; Cunningham and Ow, 1996; Chen *et al*., 2004).

In this study, the effect of reducing the time required for remediation increased Pb removals, which could be attributed to EDTA enhancement of the bioavailability, and the improvement of uptake and translocation efficiency of Pb in the shoot. Huang *et al.* (1997) indicated that EDTA was the most efficient chelator for inducing the hyperaccumulation of Pb in pea plants shoots

There are several ways in which plant growth-promoting bacteria can directly facilitate plant growth. They may fix atmospheric nitrogen and supply it to plants; which is usually a minor component of the benefit that the bacterium provides to the plant, synthesise siderophores; which can sequester iron from the soil and provide it to plant cells that can take up the bacterial siderophore–iron complex, synthesise phytohormones; such as auxins, cytokinins and gibberelins that act to enhance various stages of plant growth, and finally, they can also solubilise minerals such as phosphorus to make them more readily available for plant growth (Babu, Kim and Oh, 2013; Glick, 1995 and Click *et al*., 2007).

Furthermore, plant-associated bacteria play a key role in host adaptation to a changing environment (Sturz and Nowak, 2000). Although soil bacteria-assisted phytoremediation has been studied (Whiting *et al*., 2001; Zaidi *et al*., 2006; Dell'Amico *et al*., 2008; Abou-Shanab *et al*., 2007), there is little information on the potential of endophytic bacteria

isolated from plants grown in heavy metal-contaminated soils on the phytoremediation of heavy metal-contaminated soils. Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high stress environment of the soil (Sturz *et al*., 2000).

Citterio *et al*. (2005) reported that Microorganisms (e.g. nitrogen-fixing bacteria and mycorrhizal fungi) that enhance plant growth and increase soil nutrient content are likely to be removed from soil in the excavation process. Moreover, plant roots, soil microbes and their interaction can improve metal bioavailability in rhizosphere (Yang *et al*., 2005; Saravanan *et al*., 2007; Abou-Shanab *et al*., 2008).

Soil microorganisms can affect trace metal mobility and availability to the plant, they can produce iron chelators and siderophores for ensuring iron availability, reduce soil pH, and/or solubilise metal phosphates. Microbes influence root parameters, such as root morphology and growth and an increase in root exudation of organic solutes could affect the rate of phytosiderophore release. In turn, rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Babu, Kim and Oh, 2013; Burd *et al*., 2000; Guan *et al*. 2001).

5.4.4. Effect of Soil Amendments on Accumulation and Translocation of Pb

The mobility of heavy metals from poluted substrates being transported into the roots of the plants and its ability to translocate the metals from the roots to the harvestable parts of the plant were evaluated respectively by measuring and calculating

the bioconcentration factor (BCF) and the translocation factor (TF). The ability of plants to tolerate and accumulate heavy metals is useful for phytoextraction and phytostabilisation purposes (Yoon *et al*., 2006). Plants with bioconcentration factors greater than one and translocation factor less than one (BCF > 1 and TF < 1) have the potential for phytostabilisation (Yoon *et al*., 2006). As shown in Table 5.4, the BCF in plants with different amendments is greater than 1; which indicates that the plant used in this experiment can be used for Pb stabilisation in soil and it can prevent the occurance of leaching into groundwater (Kumar, et al 2013).

5.5. Conclusion

Phytoremediation is still in its research and development phase, with many technical issues still needing to be addressed. The results, though encouraging, suggest that further development is needed. Phytoremediation is an interdisciplinary technology that can benefit from many different approaches. Results already obtained have indicated that *Eucalyptus camadulensis* can be effective in metal remediation. The processes that affect metal availability, metal uptake, translocation, chelation were investigated in this study. As a result, it can be concluded that some amendments can be used successfully to increase the metal bioavailability. The study indicated that, without amendments, the availability of heavy metals in soil is relatively low in comparison to the addition of amendments. Comparing each individual amendment added to the soil, it was seen that the metal bioavailability increased as EDTA concentrations increased from 5 mmol to 15 mmol, increases were also seen where bacterial inoculum (*Alcaligenes eutrophus*) and

mixed amendments were added to enhance Pb phytoremediation. The results of the present pot experiment showed that *Eucalyptus camadulensis* can tolerate a wide range of Pb concentrations, and accumulate high concentrations of Pb in the above-ground parts (shoots) when amendments such as EDTA and Alcaligenes eutrophus bacteria were added. *Eucalyptus camadulensis* also has a great ability of dissolving the metal in the soil and enhancing the accumulation of Pb in shoots with 15 $mmol.kg^{-1}$ EDTA and *Alcaligenes eutrophus* bacteria. Therefore, the *Eucalyptus camadulensis* can be a suitable plant for phytoremeadtion, especially for Pb contaminated soils and the application of EDTA and *Alcaligenes eutrophus* bacteria; which can significantly increase the metal concentration in harvestable above ground parts of plants. The next chapter will discuss the recovering the lead, which is accumulated in plants during the phytoremediation process.

Chapter Six

Recovering the accumulated lead from plants during the phytoremediation process

This chapter details the methods used for recovering the accumulated lead from plants during the phytoremediation process. Firstly, an overview of the phytomining technology is offered. Then the recovering methods of the accumulated Pb from *E. Camaldeulensis* and the calculation of the feasibility of lead in the target area of the study are presented.

6.1. Introduction.

Metals could be recovered during phytoremediation process through a process known as Phytomining. Phytomining has emerged as an environmentally friendly technology that employs plants for the uptake of heavy metals (Boominathan *et al*., 2004; Brooks *et al*., 1998; Anderson *et al*., 1999). This technology involves growing plants on appropriate sites, harvesting the metal-accumulating plants, and treating biomass in order to recover metal. It has the potential to allow economic exploitation of low-grade surface ores or mineralized soils that are too metal-poor for conventional mining (Boominathan *et al*., 2004).

Phytomining is regarded as a 'green' approach compared to the environmentally sensitive and energy intensive practice of mining, involving the use of plants to extract valuable metals from both solid and liquid substrates (Brooks *et al*., 1998; Anderson et al., 1999 Robinson et al 2003). Phytomining is actually a subset of a larger field known as phytoextraction, the process of using plants to beneficially absorb mineral species from soils, sediments and ground-water. Applications of phytoextraction include phytoremediation, where non-naturally occurring contaminants are recovered for disposal or reuse and phytostabilisation, where contaminant species are immobilised *in situ* via plant action. There are numerous successful examples of plants being used to treat contaminated environments containing cadmium, copper, cobalt, mercury, lead, nickel, thallium, arsenic, selenium, cyanide, hydrocarbons residue from explosives and radioactive compounds (Salt et al., 1998; Prasad et al 2002). In contrast to phytoremediation, the objective in phytomining is to recover the mineral from sites for commercial gain (Brooks et al., 1998; Anderson et al., 1999; Robinson et al 2003).

A focal point of soil plant interactions is the micro ecosystem surrounding the plant roots, the rhizosphere, characterized by different physical, chemical, and biological conditions created by the plant roots and its surrounding soil environment. Soil solution is drawn from the roots to the above ground portions of their biomass by plant water uptake. This depends upon the root absorption factor, a dimensionless parameter describing the xylem/soil solution metal concentration gradient (Marschner, 1995; Robinson *et al*., 2003). Increased understanding of the role of metal extracting plants in the circulation of

minerals in the biosphere has made them important biotechnological tools in mining processes from low-grade ores. Plants have shown several response patterns to the presence of high metal concentrations in the soils. Most plants are sensitive to high metal concentration. While others have developed resistance, tolerance, and accumulation in roots and above ground tissues such as shoot, flower, stem, and leaves (Barcelo *et al*., 1994). The phenomenon of plants accumulating inordinate concentrations of heavy metals was termed "hyperaccumulation" (Robinson *et al*. 2003; Anderson *et al*., 2003).

A "hyperaccumulator" is defined as a plant that can accumulate metal to a concentration that is 100 times greater than ''normal" plants growing in the same environment (Baker and Brooks, 1989; Anderson *et al*., 2003). Hyperaccumulators efficiently extract metals from the metalliferous soils and then translocate them to above ground tissues. After sufficient growth, the plant is harvested and left for drying. Dried plant material is reduced to an ash (with or without energy recovery) which is further treated by roasting, sintering, or smelting methods, which allow the metals in an ash or ore to be recovered according to conventional metal refining methods, such as acid dissolution and electro winning (Figure 6.1) (Robinson *et al*., 1999). Thus, phytomining is the *in situ* removal of metals from sub-economic ore bodies or from contaminated mine sites with the additional aim of the recovery of economic amounts of metals from the plants (Chaney *et al*., 1998; Anderson *et al*., 1999).

Figure 6.1. Integrated process for bioharvesting of metal by phytomining (Robinson *et al*. 1999)

Natural metal accumulating plants release metal chelating compounds (phytochelators /phytosiderophores) to the rhizosphere, which increases the bioavailability of metals that are tightly bound to the soil and helps to carry them into plant tissues (Eapen and DSouza, 2005). Phytochelators are usually low molecular weight organic compounds, such as malic, malonic, oxalic acids, acetic acid, succinic acid, sugars, oxalic acids, amino acids and phenolics, that can change the metal speciation and thus metal bioavailability (Cieslinski *et al*., 1998; Ma *et al*., 2001; Nascimento et al., 2006). Some of the metal chelating compounds, such as mugenic acid and avenic acids, are released by the plants in response to nutrient metal deficiency, which increases the bioavailability of metals, (as is the case with iron (Ma and Nomoto, 1996)), aluminium (Pellet *et al*.,1995), and zinc (Cakmak *et al*., 1996) and helps to carry them into plant

tissues. Metal chelate complexes may also be transported across the plasma-membrane through the same process (Romheld, 1991).

6.2. Mechanism of Metal Hyperaccumulation

Metal hyperaccumulation is a complex and rare phenomemon that occurs in plant species with high metal uptake capacity. The process involves several steps as shown in Figure 6.2 below.

Figure 6. 2. Molecular mechanisms proposed to be involved in transition metal accumulation by plants (*Yang at el., 2001*)

6.2.1 Bioactivation of Trace Metals in the Rhizosphere

As stated earlier in section 5.3.3, the bioavailability and plant uptake of heavy metals in the soil is predominantly affected by metal content, pH, oxidation state of mineral components, redox potential of the system's water content, cation exchange capacity, organic substances, and other elements in the rhizosphere. The rhizosphere provides a complex and dynamic microenvironment where microorganisms, such as free living as well as symbiotic rhizobacteria and mycorrhizal fungi form unique communities. These organisms have considerable potential for detoxification of hazardous waste compounds, and their interaction can improve metal bioavailability in the rhizosphere through secretion of protons (H+ ions), organic acids, phytochelatins (PCs), amino acids, and enzymes (Figure 6.3) (Yang *at el*., 2001 Abou-Shanab *at el*., 2006).

Figure 6.3: Bioactivation of trace metals in the rhizosphere (*Yang at el., 2001*).

1. Acidification by Roots

Acidification of the rhizosphere and exudation of carboxylate are considered potential targets for enhancing metal accumulation. Secretion of H+ by roots could acidify the rhizosphere and increase metal dissolution. The proton extrusion of the roots is operated by plasma membrane H^+ ATPase and H^+ pumps (Ghosh and Singh, 2005a).

2. Secretion of Organic Acids Roots

Many hyperaccumulator plants excrete organic acids, such as malic, malonic, and oxalic acids, which act as chelators and decrease the rhizosphere pH, thus making metal cations bioavailable. The organic acids can facilitate metal uptake by plants (Huang *al et*., 1998; Ma *et al.*, 2001).

3. Secretion of Metal Chelating Compounds

Root microbe (e.g. bacteria and fungi) interaction changes the soil conditions in the rhizosphere and increases the solubility of the retained metals in the rhizosphere soil. The bioavailability of metals in hyperaccumulating plants can be enhanced by microbe secreting phytosidophores into the rhizosphere, as this chelates metals into the soil solution. Chelating agents enhance metal desorption from soil, thus increasing the bioavailability of metal in the soil solution and increase accumulation in plants (Huang e*t al*., 1998; Yang at el., 2001).

4. Rhizosphere Associated With Microorganism

Rhizosphere is populated by large concentrations of microorganisms, which in this case, mainly consist of bacteria. These root-colonizing bacteria may significantly increase the bioavailability of various heavy metal ions for uptake (Ma *et al*., 2001 and Callahan *et al*., 2006). First, they catalyze redox transformations leading to a change in soil metal bioavailability. Secondly, soil microorganisms have been shown to exude organic compounds, which stimulate bioavailability and facilitate root absorption of possible metal ions (Salt e*t al*., 1995). Other soil organisms, such as plant-growth-promoting rhizobacteria, can also contribute to plant growth and metal tolerance and enhance heavy metal accumulation by hyperaccumulators, such as Zn by *Thlaspi caerulescencens,* Ni by *Allysum murale,* and *Thlaspi goesingense* (Ma *et al*., 2001 and Idris *et al*., 2004).

6.2.2 Root Absorption and Transport to Shoot.

Soluble metals can enter into the root symplast by crossing the plasma membrane. While it is possible for solutes to travel up through the plant by apoplectic flow; a more efficient method of moving up the plant is through the vasculature of the plant, called the xylem. To enter the xylem, solutes must cross the casparian strip, a waxy coating, which is impermeable to solutes, or they may also be able to pass through the cells of the endodermis. Therefore, to enter the xylem, metals must cross a membrane, probably through the action of a membrane pump or channel. This type of transport of metals, which takes place in xylem after they cross the casparian strip, is called symplast

transport (Ma *et al*., 2001 and Idris *et al*., 2004). This route is more regulated due to the selectively of the permeable plasma-membrane of the cells that control access to the symplast by specific or generic metal ion carriers or channels (Gaymard, 1998; Hall, 2002). The flow of xylem sap will transport the metals into the shoots Yang *et al*., 2001). Several classes of proteins have been implicated in heavy metal transport in plants. These include; heavy metal or CPx-type ATPase, the natural resistance- associated macrophage protein (Nramp) family of proteins, the cation diffusion facilitator (CDF) family proteins, zinc–iron permease (ZIP) family proteins, etc. Xylem loading is operated through cation–proton antiport, cation-ATPases or ion channel (Williams *et al*., 2000; Yang *et al*., 2005).

6.2.3. Distribution, Detoxification, and Sequestration of Metal Ion

At any point along the pathway, metals can be converted to a less toxic form through chemical conversion or by complexation with organic acid such as malate, citrate, and nicotianamine (Gendre *et al*., 2007). Various oxidation states of heavy metals have very different uptake routes, transport, and detoxification characteristics in plants. Once the metals are translocated to shoot cells, they are stored in cellular locations, such as trichone (apoplast tissue), epidermis, mesophyll, cell wall, etc., where the metal will not damage the vital cellular processes (Shah *et al*., 2007). The final step for accumulation of most metals is the sequestration of the metal away from any cellular processes that it might disrupt. Metal binding proteins, such as metallothioneins (MTs) and phytochelatins (PCs), in plants play an important role in sequestration and also enhance metal tolerance and accumulation (Eapen e*t al*., 2005).

6.3. Model of a Phytomining Operation

A model of the proposed economic scheme for phytomining is shown in Figure 6.4. This system applies to either natural or induced hyperaccumulators.

Figure 6.4. The Phytomining Process (Anderson *et al*, 1999)

The economical aspects of the operation are dependent on a number of factors, such as metal content of the plant, biomass production and the energy from combustion of the biomass can be recovered and sold (Anderson *et al*, 1999).

6.4. The Economical aspects of Phytomining

The economic model of phytomining is shown in figure 6.5. To recover the accumulated metal from the biomass, there are essentially two viable techniques. The first technique is the use of a high temperature pyrolysis or combustion machine followed by smelting the ash. This is attractive because there is a possibility of being able to use the energy generated during combustion to produce electricity. The second technique relies on acid digestion of the plant matter and further processing, e.g. electrominning or solvent extraction to recover the metal (Koppolu *et al*., 2003).

Figure 6.5. Process Model for Phytomining (*Brooks et al. 1998*)

Although there are clear economic limits in terms of biomass production and metal content in respect to the potential use of any plants for phtomining, the same is not true for the wider subject of phytoremediation. Whereas, phytomining is limited by the need to produce a commercially viable metal crop, this is not the case for phytoremediation. Table 6-1 shows the elemental content (µg/g in dry matter) that would be required in plants with a fertilised biomass of 1-30 t/ha to give a gross financial total for phytomining with plants of a 10 t/ha biomass.

There are practical limits to phytomining (Brooks and Robinson 1998). The main variables that control its economic feasibility are: the metal price, the plant biomass, and the highest achievable metal content of the plant (as can be seen in Table 6.1). Metal values range from about $15 000 000 t for platinum to about $600 t for lead. At these extremes, a plant with a biomass of 20 t/ha, such as *B. coddii*, would need to contain about 1.7 mg/ g platinum or > 4% lead.

An economic phytomining system model is shown in Figure 6.2. The system differentiates between annual and perennial crops and takes into account fertilization and soil exhaustion. The success of a project depends on whether some of the energy of combustion of raw materials can be recovered. In tropical regions, it is possible to have crops maturing each month, and thus keep the incineration plant busy throughout the year. It has also been suggested (Chaney, 1998) that biomass could be stored in the field or near the incineration plant for burning according to the energy-requirement schedule.

Table 6.1. Metal Concentrations (µg g^{-1} day mass) in vegetation required to provide a total \$500 ha^{-1} return (excluding energy of incineration) on hyperaccumulator crops with varying biomass.

Induced hyperaccumulation probably required **(***Brooks et al., 1998*)

Beyond the theoretical and pilot-plant stages of phytomining, two scenarios can be investigated. The first is the development of a large-scale commercial project involving square kilometers of metal-rich soils, such as those derived from ultramafic rocks or low-grade mineralisation. The second, and perhaps more likely, scenario is phytomining by smallholders throughout a region, in which a farmer might grow a few hectares of plant material and have it collected for processing at a nearby facility. This

should be preferably close to a large city, where industrial waste could be used as feedstock for the incineration plant, which in turn could supply steam for producing local supplies of electricity. An obvious site for such small-scale phytomining is Brazil, where there are large areas of nickel mineralization and ultramafic soils from which it is uneconomic to extract metal conventionally. Farmers in Brazil are reported to have attempted and failed to grow crops such as soya bean on nickel-rich ultramafic soils (Brooks *et al*., 1992).

Figure 6.6: High technology for recovering the lead accumulated from plants

6.5. Recover the Accumulated Pb from *E. Camaldeulensis* in Azzawiyah City

There are many debates regarding the fate of harvested heavy metals hyperaccumulator plants. Industrially, plants may be incinerated, composted, or chemically treated to leach out the heavy metals. It could also be carefully composted before disposal, but only in a separate, enclosed area, to prevent the lead from leaching out as the plants break down (Anderson *et al*., 2003).

It should also appeal to mining companies because it offers possibilities to exploit and extract ores from plants or mineralised soils that are uneconomical to develop by conventional methods. The comparative advantages of phytomining make it a technology worth investigating in as a sustainable alternative to traditional mining methods, such as wood strode, wood shreds, gas burner, separation and re-use heavy industry (Anderson *et al*., 2003).

6.5.1. The feasibility of Pb phytomining in Azzawiyah city

The calculations in (Table 6.2) assumed a soil lead concentration of 840 mg/g estimated that the base case alternative to lead phytomining of serpentine soils was *Eucalyptus sp*, which has an annual value of 460 US$/ha, and thus to be viable, Pb phytomining must be more profitable than this type of land use. This requires that the minimum extractable lead concentration in *Eucalyptus sp* 2300 mg/g, which will result in an uptake in the plant biomass and 70 kg/ha of extracted lead.

Table 6.2: Concentration of lead (Pb) that extractable by plants \ha to provide a crop with a gross value of $ per hectare.

Amendments	Growth period days	Total metal in the harvestable part plant mg	Number of plants \ ha	Total metal by extractable \hakg	Price of metal $
Eucalyptus sp **+ composite 5%**	**90**	**1400**	**50000**	**70**	**280**
Eucalyptus sp **+ composite 10%**	**90**	**1450**	**50000**	**72.5**	**290**
Eucalyptus sp **+ composite 25%**	**90**	**1510**	**50000**	**75.5**	**302**
Eucalyptu s sp**+ EDTA 5 mmol**	**90**	**1600**	**50000**	**80**	**320**
Eucalyptus sp**+ EDTA 10 mmol**	**90**	**1700**	**50000**	**85**	**340**
Eucalyptus sp **+ EDTA 15 mmol**	**90**	**2300**	**50000**	**115**	**460**
Eucalyptus sp **+ Hoagland 25%**	**90**	**1400**	**50000**	**70**	**336**
Eucalyptus sp **+ Hoagland 50%**	**90**	**1476**	**50000**	**73.8**	**295.2**
Eucalyptus sp **+ Hoagland 100%**	**90**	**1495**	**50000**	**74.7**	**299**
Eucalyptus sp **+ Bacterial inoculum's**	**90**	**2200**	**50000**	**110**	**440**
Eucalyptus sp **+ Bacterial mixed**	**90**	**1600**	**50000**	**80**	**320**

In the instance, however, where phytomining occurs on land immediately adjacent to a former smelter, then the opportunity cost of the land is like ly to be negative, because the land requires extensive remediation prior to being used for another activity. The data in Table 6.2 were calculated using the results in Chapter 6, Section 6.3.3.

6.6. Conclusion

This chapter discussed the *phytomining* methods used to recover the accumulated lead from plants during the phytoremediation process. It provided some calculation of lead (Pb) that can be extracted by plants using different amendments. It was assumed that the highest extractable lead concentration was 115 kg/ha and 110 kg/hs in *Eucalyptus sp* + EDTA 15 mmol and *Eucalyptus sp* + Bacterial inoculum, where the estimated costs were 460 US$/ha and 440 US$/ha.

It can be argued that *phytomining* method *is not* worth in recovering lead from plants because lead element is not valuable metal. Thus, such method can be used with different metals, such as gold. Furthmore, in Libya, phytomining method can used to recover other metals that near oil fields and gas production companies, or any other industrial factories. If phytomining was used to recover the lead from plants, it is suggested that the traditional method (e.g. wood strode, wood shreds, gas burner, separation and re-use heavy industry) would be worth as it mimumize the costs of recovery of method. Next will be the general discussion of the present study findings.

Chapter Seven

Discussion

7.1. General discussion

In chapter seven, a general discussion of the whole findings of the present study will be presented. This is followed by the calculations of the economical benefits of the methods used in this study. These include: calculation of the weight of the studied area, cost of Hoagland solution required for the studied area, cost of EDTA required for the studied area, cost of compost required for the studied area, and finally cost of Bacteria required for the studied area.

Phytoremedation is a way to produce energy from plant. It is a cost effective and environmentally friendly approach, and is built on two main strategies: phytoextraction and phytostabilization. Recently, many researchers have started focused on hyperaccumulators which accumulate 10 to 100 times more heavy metals than non hyperccumulators and encompass the contaminant in to their biomass. In order to determine how to make phytoremediation run more efficiently, the use of crops and plants with high biomass production rates have been investigated with the addition of soil amendments. Any amendments added have to be tested to ensure a more effecive and feasible phytoextraction strategy.

In this thesis, heavy metals have been considered as one of the main pollutants responsible for environmental contamination due to their high toxicity and persistence in

the environment. The persistence of heavy metals in soil is as a result of the binding of heavy metals to organic and inorganic soil constituents, and their presence as insoluble precipitates. Consequently, a large proportion of heavy metals are not available for root uptake by field-grown plants. To encourage phytoremediation in the field, methods for increasing heavy metals' phytoavailability in soil and its transport to plant roots are vital (Ernst, 1996; Kukier *et al*., 2004).

Moreover, heavy metal bioavailability in the soil rhizosphere is another critical factor that determines the efficiency of metal translocation and phytostabilization process (Ma *et al*., 2011). The mobility and availability of soil heavy metals to plant roots can be affected by microbial activities, such as; acidification processes, the release of chelators and redox changes (Smith and Read, 1997; Abou-Shanab *et al*., 2003a). Microorganisms, in association with roots, found in the rhizosphere, form unique communities that have considerable potential for the detoxification of toxic compounds such as heavy metals (Black *et al*., 1993; De-Souza *et al*., 1999). The success of phytoremediation is dependent on the potential of plants to yield high biomass and withstand heavy metal stress. Hence, the improvement of the interactions between plants and beneficial rhizosphere microorganisms will not only enhance biomass production but also encourage the tolerance of plants to heavy metals, and is therefore considered to be an important component of phytoremediation technologies (Glick, 2003).

Vehicular emissions, industrial discharge and other man-made activities are the main sources of heavy metals present in the environment. Previous and current studies

indicate that roadside soils and plants are generally contaminated with heavy metals, in particular Pb and Cd, as a result of vehicular emissions (Singh *et al.*, 1997; Liu *et al*., 2007). Also, vehicular emissions can change soil quality parameters, including metal concentrations (Ramakrishnaiah and Somashekar, 2002). The contamination of roadside soil with Pb and Cd can be affected by traffic load variations and are consistent with findings reported by Fakayode and Olu-Owolabi (2003). Morton-Bermea *et al*. (2009), indicated that the distribution of Pb and Cd in roadside soils was highly affected by the distance and density of traffic on roads (Bakirdere and Yaman, 2008).

Lead (Pb) one of the elements of major concern in environmental heavy metals pollution in Libya, exhibited high levels of contamination closer to highways. This occurrence is attributed to the emission of Pb particulate matter emitted from petrol in automobiles, as the fuel used by automobiles in Libya is still mostly leaded. These particulate emissions settle not far from the roadside thereby contaminating the surrounding soil and vegetation. In this study, soils near roads having high density of traffic were highly contaminated with Pb, and as the distance from the road increased, Pb levels decreased. Also, results showed that the heavy metal content in plant tissues (*Doedonea viscose*) collected from sites close to the roadside was relatively high compared to the same plant species collected from agricultural fields. These results clearly indicated that the variation in concentrations of Pb along the different sites on the roadside might be due to variations in traffic density (Oncel *et al.*, 2004).

In this study, experiments were undertaken to provide new aspects and strategies for the phytoremediation of heavy metals in soil. Experiments involved the use of different plants and novel amendments. Three plants were selected and were examined in order to discover which one was a hyperaccumulator plant. The Plant species; *Eucalyptus camaldeulensis*, *Medicago sativum*, and *Brassica juncea*, were chosen based on their high biomass and ability to remove heavy metals from contaminated sites (Beladi *et al*., 2011; Waranusantigul *et al*., 2011).

The results showed that the Pb contaminated soil had negative effects on the shoot and root of both the fresh and dry weight of all three plants *(Eucalyptus camaldeulensis, Medicago sativum, and Brassica juncea*), this negative effect was also reported by Chen and Cutright (2001), Hajiboland (2005) and Tlustos et al. 2006 on hyperaccumulator plants. The ability of plants to tolerate and accumulate HMs is useful for phytoextraction and phytostabilization purposes. The process of phytoextraction generally requires the translocation of HMs to the easily harvestable plant parts, such as the plant shoot (Yoon *et al*., 2006). Yoon et al., (2006) also reported that of phytoremedation can be phytostabilization or phytoextraction depends on BCFs and TFs. Pb concentration in the root and shoot of three plants grown in lead contaminated soil was greater than in those grown in contaminated soil.

Therefore, among the tested plant species, *E. camadulensis* was suitable for phytoextraction of Pb and Zn, while *M. sativum* and *B. juncea* were suitable for phytostabilization of Cu and Cr. This study showed that *E camaldulensis* has the ability

to translocate and accumulate Pb into the shoot. These results are also corroborated by Neman *et al*., (2012), who reported that *E.camaldulensis* have massive shoot systems which are able to accumulate high concentrations of heavy metals. Also, *E. camadulensis* is recognized as an appropriate tree for high biomass production. *E. camadulensis* also proved to be highly adaptable to some of the local environmental difficulties experienced, such as; drought periods, high ambient temperatures and revealing fast growing abilities compared to *M. sativum* and *B. Juncea*. Therefore, *E. camadulensis* was selected to assist in the investigation of increasing the efficiency of phytoremediation. The experiment used different treatments and assessed how efficient each treatment was. The different treatments tested on the plants were: compost (0%, 5%, 10%, and 25%), EDTA (0 mmol, 5 mmol, 10 mmol, and 15 mmol), Hoagland solution (0%, 25%, 50%, and 100%), Bacterial inoculum (Alicaligenes eutrophus) and mixed amendments (5% Composite + 5 mmol EDTA + 25% Hoagland solution + bacterial inoculum).

All EDTA treatments increased Pb accumulation in *E. camadulensis* shoot but the highest pb accumulation was recorded when 15mmol of EDTA was used. This result was supported by findings by Kumar et al., (2011) with *B. juncea*. The bulk of soil metal is commonly found as insoluble compounds not available to be transported into the roots; which consequently affects the metal uptake of hyper-accumulating plants. However, recently, many synthetic chelators, such as EDTA, have been applied to Pb-contaminated soils to increase the mobility and bioavailability of Pb, thereby increasing the amount of accumulated Pb in the aerial parts of phytoextracting plants (Luo *et al*., 2006; Turgut *et*

al., 2005). The results for EDTA are in agreement with the view that EDTA is the most efficient chelating agent required to increase Pb accumulation in plant shoots (Luo *et al*., 2006). EDTA has a strong ability to enhance phytoextraction of Pb and was more effective in terms of solubilising soil Pb for root uptake and its translocation to above-ground parts, due to its strong chemical affinity for Pb.

In this study, EDTA was found to enhance bioavailability and improve the uptake and translocation of Pb into the shoots. Also, EDTA was the most efficient chelator for inducing the hyperaccumulation of Pb in *E. camadulensis* plants shoots. Furthermore, lowering soil pH can enhance the efficiency of chelating agents on metal solubilization and accumulation. Soil microorganisms can affect trace metal mobility and availability to the plant, they can produce heavy metal chelators and siderophores in order to ensure heavy metal availability, reduce soil pH, and/or solubilise metal-phosphates (Abou-Shanab *et al*., 2003; 2006).

Soil pH is also an important factor for Pb adsorption and desorption in soils: a decrease in pH increases Pb desorption from soil components, resulting in an increased Pb concentration in soil solution. Using *Alcaligenes eutrophus* amendmend as an amendment can play an important role in altering the rhizosphere environment, and consequently effecting soil pH levels. The change in pH was key in the conversion of heavy metals from being unavailable to available; subsequently, the phytoextraction of heavy metals is increased.

Bacterial inoculations lead to an increase in Pb accumulation in the shoot by 1.3-fold higher than the control, and this can be explained by the fact that rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Burd *et al*., 2000; Guan *et al*., 2001). Ma et al., (2011) reported that the role of microbiota, specifically rhizopheric microorganisms, in the development of phytoremediation techniques has to be expounded in order to speed up the process and optimize the rate of mobilization/absorption of pollutants such as heavy metals. Therefore, the application of microbe-mediated processes could be a promising alternative to chemical amendments, whereby microbial metabolites/processes in the rhizosphere affect plant metal uptake by altering its mobility and bioavailability (Wenzel, 2009; Glick, 2010; Rajkumar *et al*., 2010; Ma *et al*., 2011; Miransari, 2011; Aafi *et al*., 2012; Yang *et al*., 2012).

The benefits of microbes in phytoremediation, as opposed to chemical amendments, include their ability to proliferate in-situ in rhizosphere soils, and to biodegrade microbial metabolites that are less toxic. In addition, plant growth-promoting substances (such as siderophores), plant growth hormones (1-aminocyclopropane-1-carboxylic acid (ACC) deaminase) produced by plant-associated microbes improve the growth of the plant in metal contaminated soils (Lebeau *et al.,* 2008; Glick, 2010; Kuffner *et al*., 2010; Rajkumar *et al*., 2010; Babu and Reddy, 2011; Luo *et al*., 2011; Ma *et al*., 2011; Wang *et al*., 2011; Luo *et al*., 2012).

Microbial activities in the root, rhizosphere and soil enhances the effectiveness of phytoremediation processes in heavy metal contaminated soil in two ways: (i) Direct promotion of phytoremedation in which plant associated microbes enhance translocation thereby facilitating phytoextraction, or reducing the mobility/availability of metal contaminants in the rhizosphere (phytostabilization) and (ii) Indirect promotion of phytoremediation in which the microbes confer plant heavy metal tolerance and/or enhance plant biomass production in order to remove/arrest the pollutants . Rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Burd *et al*., 2000; Guan *et al.,* 2001).

Alcaligenes eutrophus CH34, used in this study, and related bacteria are adapted to survive in environments with high concentrations of heavy metal ions (Diels and Mergeay 1990; Abou-Shanab *et al*., 2005). In general, these processes can either solubilize metals, thereby increasing their bioavailability and potential toxicity, or immobilize them thereby reducing the bioavailability of metals. An exploitation of the processes described above is therefore necessary in the bioremediation of heavy metal contaminated soils (Lovley and Coates, 1997; Gadd, 2000; Barkay and Schaefer, 2001; Lloyd and Lovley, 2001; Abou_Shanab *et al*., 2007; 2008).

Overall, mixed amendments, bacterial inoculum and 15 mmol EDTA have been shown to enhance the solubilization of Pb and significantly increase the rate of metal translocation from the root to shoot in *E. camaldeulensis* grown on Pb contaminated soil,

with *Alicaligenes eutrophus* followed by 15mmol/kg EDTA giving the best results, in the treatment of Pb contaminated soils.

7.2. The Economical benefits of the methods used

7.2.1. Calculation of the weight of the studied area

The weight by 1/4 can be calculated based on standard acre furrow slice, which is as follows:

100m * 60*0.18m =1080m^3.

The density of sand soil =1.6gcm^3 → 1.6 ton/m^3.

d= m/v → m= d*v

d= density of soil

m = mass of soil

v= volume of soil

mass of soil = 1080m^3* 1.6 ton/m^3 = 1728 ton .

7.1.2. Cost of Hoagland solution required for the studied area

25% of Hol =648.000L * 4$= 2,592,000$

50% of Hol = 1.296.000L*4$=5,184,000$

100% of Hol = 2.592.000L*4$=10,368000$

7.2.3. Cost of EDTA required for the studied area

5mmol = 6.480.000L*2.7$= 17,496,000$

10 mmol = 12.960.000L * 2.7$= 34,992,000$

15mmol =19440.000L*2.7= 52,488,000$

7.2.4. Cost of compost required for the studied area

5 %compost = 86.40 ton * 70$= 6,048 $

10% compost = 172.80 ton* 70$=12,096 $

25 compost = 432 ton* 70$ =30,240 $

7.2.5. Cost of Bacteria required for the studied area.

The cost of of nutrient broth = 43.200 L/38.4L= 1125

The cost of nutrient broth= 1125 * 65$ = 73,125 $

Total cost of bacteria with nutrient broth = 73.125 + 300$ =73,425$

The present study showed that using *Alcaligenes eutrophus* is better than using EDTA because the use of *Alicaligenes eutrophus* is more economically viable as shown above by its low costs in comparison to all other treatments. EDTA chelators have many

advantages, such as increased accessibility and consequently phytoextraction of heavy metals by plants, however it may increase leaching of heavy metals through soil solution to ground water and to other sites by lateral movements. Also, plant growth may also be affected by chelates.

Other adavantage of *Alicaligenes eutrophus* is that it can be adapted as amendment of soil because of its efficacy and low costs. Moreover, it is more economically to other conventional technologies, as the estimated cost of *Alicaligenes eutrophus* in this study is 42.49 $/ton, comparing with the study condacted by Glass (1999). It was foud that the soil washing cost was between 120 –200$/ton, and stabilization cost was between 50 -330$/ton, and chemical treatment cost was between 100- 500$/ton (Glass, 1999).

As it has been mentioned in chapter three, the target area of investigation is contimenated by heavy metal, especially lead element. In addition, the treatment that will be used to uptake the lead from the soil is phytoextraction, which was planed to last approximately five years according to Azzawiyah city council. However, this plan seems to be impossible to put into practice now because of the political situation in Libya. Nevertheless, if the political situation is improved in the country, this method will be applied, and the area will be developed and used for public investment.

Chapter Eight

Conclusion, recommendations and future work

This chapter covers three important elements of the present study. Firstly, a conclusion of the thesis is presented, followed by the contribution of the study. Secondly, the researcher's recommendations and suggestion for further work is highlighed. Finally, the implications for further research is outlined.

8.1. Conclusion

Heavy metal pollution of soil and water is a significant environmental problem and has a negative impact on human health and agriculture. Until now, methods used for their remediation, such as physical separation, acid leaching or electrochemical processes are not suitable for practical applications, because of their high cost and low efficiency. Thus, the development of remediation strategies for heavy metal-contaminated soils has been necessary.

o Phytoremediation is an emerging technology for the remediation of heavy metal contaminated soil, which requires more information of hyperaccumulator species, especially for specific metals. The result of this study revealed that some metal concentrations, such as Pb, Zn and Cd are decreased with the increase the distance from the roadside. Thus, the level of Pb was higher than other metals.

- The level of Pb concentration in sample 1 (3mS) was higher (840mgkg-1) than the standard level (Kabata-Pendias and Pendias, ICRCL trigger value, and Dutch list)

- Accumulation of metal in the soil and subsequent transfer to plants growing along the edge of the road could occur as a result of continual usage of the road by automobiles.

- The study also found that Doedonea viscose plants that were found growing in the study area were not suitable plant for heavy metal accumulation.

- Three plants (*E. camaldeulensis, Brassica napus* and *Medicago sativum*) used for phytoremediation in this study indicated that shoot and root dry weights were different in contaminated soil and control soil.

- Pb, Zn, Cu and Cr concentrations in the root and shoot of the three plants mentioned above, which were grown in contaminated soil, were higher than the same plants grown in control soils.

- Considering the translocation factor (TF) and bioconcentration factor (BCF), *E. camadulensis* was suitable for phytoextraction of Pb and Zn, while M. sativum and *B. juncea* was suitable for phytostabilization of Cu and Cr.

- Amendments (e.g. compost, EDTA, Hoagland solution and bacterial inoculum) were used to increase the bio-availability of heavy metal in soil contaminant in this study. It was found that the higher concentrations of available Pb (17.6 ± 0.68 mg/kg^{-1}) were obtained from soil amended with 15 mmol of EDTA, 17.2 ± 0.98 mg/ kg^{-1}of mixed amendment, and 15.4 ± 0.66 mg/ kg^{-1}of bacterial inoculum.

- The data revealed that all applied amendments enhanced the fresh shoot weight. It also shows that mixed amendment was the most effective treatment followed by Hoagland solution 100%. Furthermore, mixed amendment was the most effective treatment that increased the dry root weight followed by *Alcaligenes eutrophus* bacteria.

- The highest accumulation of Pb (1073±7.6 mg kg-1DW) in plant root was obtained by plants cultivated in soil, which was inoculated with *Alcaligenes eutrophus*. Bacteria and ETDA 15mmol were the only treatments that increased the level of Pb in the shoot greater than the untreated control. These results show that bacteria and EDTA play an important role in increasing Pb availability in soil, thus enhancing metal accumulation by *E. camaldeulensis*.

- In this study, it is also found that the cost of Alicaligenes eutrophus was the lowest of all amendments used in this research.

- The phytomining was not suitable for the lead element because it is not valuable metal. This method can be used for extracting expansive metal, such as cold.

8.2. Contribution of the study

The contributions of this study to the science of soil contaminated and phytoremediation field in Azzawiyah city area include:

- The concentration levels of lead, cadmium and zinc decreased with the increase distance from Azzawiyah roadside. This is one the very few studies conducted in

other countries that has determined the levels of heavy metals near to the roadsides.

- According to the author's knowledge, this study was the first study used phytoremediation method in Libya.
- *E. camadulensis, M. sativum* and *B. juncea* plants have been found suitable for phytoremediation of heavy metals.
- *E. camadulensis* was found as the best suitable plant to uptake the lead from the soil, which has been recently applied in phytoremediation method.
- Some amendments were showed increase of bio-mass and accumulation of lead elements, especially, 15mmol EDTA and *Alcaligenes eutrophus.*
- *Alicaligenes eutrophus* increases the accumulation of lead element in the plants root and shoot.

8.3. Recommendations and suggested future work

This plant *E. camaldeulensis* plant is therefore recommended as appropriate for phytoremediation. The research confirmed that the 15 mmol EDTA, *Alcaligenes eutrophus* or a combination of both are effective in improving the performance of phytoremation. This study confirmed the superior performance of *Alcaligenes eutrophus* En as it increased the accumulation of lead with the *E. camaldulensis* plants.

The following ideas are suggested for further study:

- Determine and examine the bio-availability of airbourne particulates derived from vehicle emissions. Air samples should be collected from the field sites and analysed for potentially toxic elements. The effect of the particulates on human health could be considered by examining the reaction with synthetic lung fluids.
- Select optimal genotypes of *E. camaldulesis* and to initiate a program of seed multiplication.
- Determine the heavy metals concentration in plants pots before and after the experiment.
- Determine the mechanism of hyperaccumulation plants that could uptake metals from soils.
- Isolation and identification of lead tolerant bacteria from *E.camaldulensis* plant. This can be done by using 16S r DNA sequencing method. There are at least three Pb tolerant bacteria that can be isolated by media amended with different concentration, which can then be identified by PCR analysis and sequencing.
- It is suggested that other plants, which grow in Libya, could be used for phytoremediation.
- It also recommended that other elements should be investigated in other roadsides in Libya.
- The present research suggests that other studies should be conducted to investigate heavy metal in other plants which grow near highway; about 10m closer to the roads.

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Appendices

Appendix 1a. Total results of the heavy metal analysis for soil samples obtained from the target area of study

Site	Profile	Co mg kg^{-1}				Cr mg kg^{-1}			
		T	P-value	E	P-value	T	P-value	E	P-value
1(m S)	0-10	8.67^{a}	<0.01	0.10^{a}	<0.01	38.08^{a}	<0.01	0.47^{a}	<0.01
1	10-20	6.60^{ab}	0.22	0.08^{b}	<0.01	30.70^{a}	<0.01	0.38^{ab}	<0.01
2(10 m S)	0-10	11.45^{cd}		0.15^{cd}		62.32^{a}		0.77^{a}	
2	10-20	6.78^{b}		0.08^{b}		48.60^{a}		0.60^{b}	
3(3 m S)	0-10	11.70^{a}		0.14^{a}		63.12^{a}		0.57^{a}	
3	10-20	5.50^{ab}		0.06^{b}		60.34^{a}		0.76^{b}	
4(10m S)	0-10	8.91^{b}		0.11^{b}		60.09^{a}		0.75^{a}	
4	10-20	4.65^{ab}		0.06^{b}		30.83^{a}		0.38^{a}	
5(3m S)	0-10	5.98^{cde}		0.07^{cde}		55.70^{b}		0.63^{a}	
5	10-20	4.25^{ab}		0.06^{b}		34.87^{b}		0.43^{c}	
610 m S)	0-10	10.30^{cdef}		0.12^{cedf}		63.00^{a}		0.78^{a}	
6	10-20	4.95^{a}		0.06^{b}		40.01^{a}		0.50^{b}	
7(3 m N)	0-10	7.52^{defg}		0.10^{defg}		42.83^{b}		0.53^{b}	
7	10-20	4.01		0.05^{a}		40.55^{b}		0.50^{cd}	
8(3 m N)	0-10	18.20^{c}		0.22^{c}		47.90^{bc}		0.93^{bcde}	
8	10-20	6.67^{ab}		0.08^{a}		45.48^{b}		0.57^{e}	
9(3 m N)	0-10	9.85^{g}		0.12^{g}		53.31^{bc}		0.64^{bc}	
9	10-20	5.49^{ab}		0.06^{g}		38.04^{b}		0.45^{cd}	
10(3m N)	0-10	12.30^{cde}		0.15^{cde}		46.15^{bc}		0.55^{bcde}	
10	10-20	6.02^{ab}		0.07^{a}		45.00^{b}		0.55^{de}	
11(3m N)	0-10	6.95^{g}		0.08^{g}		35.01^{cde}		0.42^{cde}	
11	10-20	4.05^{g}		0.05^{a}		28.11^{c}		0.33^{f}	
12(3m S)	0-10	9.78^{g}		0.12^{g}		30.95^{de}		0.36^{de}	
12	10-20	5.50^{ab}		0.06^{g}		25.66^{c}		0..31^{f}	
13(3m S)	0-10	8.76^{g}		0.10^{g}		29.73^{de}		0.36^{de}	
13	10-20	4.89^{ab}		0.06^{g}		25.26^{c}		0.31^{f}	
14(10mS)	0-10	13.12^{g}		0.16^{g}		29.55^{de}		0.36^{de}	
14	10-20	6.52^{ab}		0.07^{g}		24.78^{c}		0.31^{f}	
15(10mS)	0-10	6.90^{g}		0.08^{g}		29.59^{e}		0.37^{e}	
15	10-20	4.21^{ab}		0.05^{g}		24.12^{c}		0.30^{f}	
16(10mN)	0-10	11.95^{efg}		0.14^{efg}		60.34^{b}		0.82^{b}	
16	10-20	5.77^{ab}		0.07^{a}		44.45^{b}		0.67^{de}	
17(10mN)	0-10	8.89^{c}		0.11^{c}		47.84^{bc}		0.59^{bcde}	
17	10-20	5.02^{ab}		0.06^{a}		41.03^{b}		0.43^{e}	
18(10mN)	0-10	13.34^{fg}		0.16^{fg}		50.20^{bc}		0.66^{bcd}	
18	10-20	6.65^{ab}		0.08^{a}		45.57^{b}		0.50^{de}	
19(10mN)	0-10	22.20^{cde}		0.27^{cde}		43.03^{bc}		062^{bcde}	
19	10-20	7.68^{ab}		0.09^{a}		40.21^{b}		0.48^{e}	
20(10mN)	0-10	10.31^{g}		0.12^{a}		34.34^{cde}		0.48^{cde}	
20	10-20	5.50^{ab}		0.06^{a}		29.76^{c}		0.40^{f}	

a , b ,c , d ,e mean don't share the same letter are significantly different

Appendix 1b. total results of the heavy metal analysis for soil samples obtained from the target area of study

site	Profile	Cu mg kg^{-1}				Ni mg kg^{-1}			
		T	P-value	E	P-value	T	P-value	E	P-value
1(3m S)	0-10	45.08^{a}	<0.01	0.56^{a}	<0.01	18.59^{de}	<0.01	0.25^{de}	<0.01
1	10-20	42.36^{a}	<0.01	0.50^{a}	<0.01	17.46^{def}	<0.01	0.20^{ef}	<0.01
2(10 m S)	0-10	40.45^{a}		0.47^{a}		20.71^{abc}		0.26^{abc}	
2	10-20	35.66^{ab}		0.35^{ab}		20.01^{a}		0.30^{a}	
3(3 m S)	0-10	50.71^{a}		0.62^{a}		19.64^{de}		0.24^{de}	
3	10-20	34.23^{abc}		0.36^{abc}		18.03^{de}		0.22^{ef}	
4(10m S)	0-10	30.60^{cde}		0.38^{cd}		20.68^{cde}		0.27^{cde}	
4	10-20	30.01^{ghij}		0.37^{hi}		19.78^{bcd}		0.25^{c}	
5(3m S)	0-10	31.96^{bcd}		0.40^{bcd}		17.87^{a}		0.20^{a}	
5	10-20	30.05^{efg}		0.39^{gh}		17.65^{a}		0.23^{a}	
610 m S)	0-10	27.00^{abc}		0.35^{abc}		16.88^{ab}		0.21^{ab}	
6	10-20	26.50^{def}		0.40^{fg}		16.59^{abc}		0.20^{b}	
7(3 m N)	0-10	30.06^{cde}		0.37^{cd}		17.60^{de}		0.23^{de}	
7	10-20	29.61^{hij}		0.38^{hij}		17.01^{def}		0.26^{ef}	
8(3 m N)	0-10	31.69^{de}		0.41^{de}		15.16^{bcd}		0.20^{bcd}	
8	10-20	25.84^{j}		0.35^{j}		14.89^{ab}		0.25^{ab}	
9(3 m N)	0-10	30.32^{de}		0.36^{de}		17.59^{de}		0.30^{de}	
9	10-20	30.01^{fghi}		0.47^{hi}		16.40^{def}		0.25^{f}	
10(3m N)	0-10	28.91^{e}		0.36^{e}		19.03^{de}		0.31^{de}	
10	10-20	26.70^{j}		0.37^{ij}		19.00^{de}		0.27^{cde}	
11(3m N)	0-10	24.95^{a}		0.35^{a}		20.05^{e}		0.25^{e}	
11	10-20	24.91^{abc}		0.41^{bcd}		16.45^{hi}		0.20^{g}	
12(3m S)	0-10	26.00^{a}		0.40^{a}		20.01^{e}		0.23^{e}	
12	10-20	25.50^{cde}		$0..45^{ef}$		18.5^{fghi}		0.20^{g}	
13(3m S)	0-10	30.70^{a}		0.36^{a}		21.10^{e}		0.28^{e}	
13	10-20	30.66^{bcd}		0.35^{def}		17.98^{fgh}		0.25^{g}	
14(10mS)	0-10	31.95^{a}		0.37^{a}		20.00^{e}		0.30^{e}	
14	10-20	30.86^{def}		0.38^{fg}		17.78^{ghi}		0.23^{g}	
15(10mS)	0-10	25.85^{a}		0.60^{a}		16.99^{e}		0.31^{e}	
15	10-20	25.01^{cde}		0.54^{def}		17.02^{fgh}		0.27^{g}	
16(10mN)	0-10	28.64^{de}		0.51^{de}		15.11^{e}		0.31^{de}	
16	10-20	26.55^{j}		0.48^{ij}		14.03^{efg}		0.29^{g}	
17(10mN)	0-10	40.99^{de}		0.45^{de}		16.86^{cd}		0.40^{cd}	
17	10-20	38.56^{j}		0.40^{j}		14.55^{cde}		0.30^{cd}	
18(10mN)	0-10	37.01^{e}		0.36^{e}		18.07^{de}		0.32^{de}	
18	10-20	36.50^{j}		0.45^{ij}		17.51^{def}		0.20^{ef}	
19(10mN)	0-10	30.21^{e}		0.38^{e}		20.00^{de}		0.31^{de}	
19	10-20	28.75^{j}		0.36^{ij}		18.49^{de}		0.20^{def}	
20(10mN)	0-10	24.55^{ab}		0.31^{ab}		20.56^{e}		0.45^{e}	
20	10-20	24.30^{abc}		0.30^{cde}		19.80^{i}		0.30^{g}	

Appendix 1c. total results of the heavy metal analysis for soil samples obtained from the target area of study

Site	Profile	Fe mg kg^{-1}				Cd mg kg^{-1}			
		T	P-value	E	P-value	T	P-value	E	P-value
1(3m S)	0-10	1092.01^{fg}	<0.01	11.40^{fg}	<0.01	0.64^{a}	<0.01	ND	
1	10-20	980.52^{a}	0.434	10.35^{b}	<0.01	0.48^{ab}	<0.01	ND	
2(10 m S)	0-10	1200.30^{efg}		10.91^{efg}		0.46^{b}		ND	
2	10-20	950.76^{a}		10.64^{b}		0.21^{def}		ND	
3(3 m S)	0-10	1610.03^{b}		16.96^{b}		0.38^{b}		ND	
3	10-20	1200.64^{a}		12.61^{b}		0.36^{def}		ND	
4(10m S)	0-10	1610.83^{b}		12.60^{b}		0.37^{b}		ND	
4	10-20	1305.03^{a}		13.73^{b}		0.25^{cdef}		ND	
5(3m S)	0-10	1287.44^{def}		13.50^{defg}		0.36^{b}		ND	
5	10-20	890.71^{a}		9.37^{b}		0.10^{cdef}		ND	
610 m S)	0-10	1320.90^{def}		13.82^{defg}		0.27^{b}		ND	
6	10-20	887.05^{a}		9.35^{b}		0.10^{f}		ND	
7(3 m N)	0-10	1415.84^{bcd}		14.90^{bcde}		0.46^{b}		ND	
7	10-20	1001.28^{a}		10.53^{b}		0.24^{a}		ND	
8(3 m N)	0-10	1502.76^{bcd}		15.86^{bcd}		0.36^{b}		ND	
8	10-20	1100.03^{a}		11.59^{b}		0.20^{abc}		ND	
9(3 m N)	0-10	1897.11^{a}		19.67^{a}		0.31^{b}		ND	
9	10-20	1500.96^{a}		15.74^{b}		0.10^{cdef}		ND	
10(3m N)	0-10	1088.73^{g}		11.51^{g}		0.16^{b}		ND	
10	10-20	761.22^{a}		8.0^{b}		0.10^{ab}		ND	
11(3m N)	0-10	1333.58^{def}		14.03^{def}		0.18^{b}		ND	
11	10-20	880.01^{a}		9.36^{b}		0.11^{ab}		ND	
12(3m S)	0-10	1596.50^{bcd}		16.88^{bc}		0.24^{b}		ND	
12	10-20	901.23^{a}		9.54^{b}		0.10^{abc}		ND	
13(3m S)	0-10	1395.84^{bcd}		14.70^{bcde}		0.20^{b}		ND	
13	10-20	1336.79^{a}		38.11^{a}		0.13^{abcd}		ND	
14(10mS)	0-10	1457.48^{bcd}		15.32^{bcd}		0.16^{b}		ND	
14	10-20	636.60^{a}		6.75^{b}		0.11^{bcdef}		ND	
15(10mS)	0-10	1365.80^{cde}		14.38^{cde}		0.16^{b}		ND	
15	10-20	900.05^{a}		9.46^{b}		0.14^{abcd}		ND	
16(10mN)	0-10	1415.34^{bcd}		14.89^{bcde}		0.40^{b}		ND	
16	10-20	995.81^{a}		10.45^{b}		0.25^{cdef}		ND	
17(10mN)	0-10	1495.56^{bcd}		15.78^{bcd}		0.30^{b}		ND	
17	10-20	1087.03^{a}		11.47^{b}		0.16^{abcd}		ND	
18(10mN)	0-10	1889.18^{a}		19.80^{a}		0.27^{b}		ND	
18	10-20	1489.40^{a}		15.61^{b}		0.10^{ef}		ND	
19(10mN)	0-10	1089.29^{fg}		11.53^{fg}		0.16^{b}		ND	
19	10-20	824.39^{a}		8.66^{b}		0.10^{abc}		ND	
20(10mN)	0-10	1338.06^{de}		14.02^{de}		0.16^{b}		ND	
20	10-20	879.80^{a}		9.25^{b}		0.10^{bcdef}		ND	

a , b ,c , d ,e mean don't share the same letter are significantly different.

Appendix 1d. total results of the heavy metal analysis for soil samples obtained from the target area of study

Site	Profile	Pb mg kg^{-1}				Zn mg kg^{-1}			
		T	P-value	E	P-value	T	P-value	E	P-value
1(3m S)	0-10	840.40^{a}	<0.01	8.80^{a}	<0.01	98.50^{a}	<0.01	1.20^{a}	<0.01
1	10-20	460.52^{a}	<0.01	8.01^{a}	<0.01	55.20^{ab}	<0.01	0.65^{ab}	<0.01
2(10 m S)	0-10	673.02^{d}		8.45^{d}		67.49^{cd}		0.80^{cd}	
2	10-20	421.35^{c}		8.22^{c}		40.31^{cd}		0.68^{de}	
3(3 m S)	0-10	806.14^{b}		8.40^{b}		90.01^{ab}		1.05^{ab}	
3	10-20	450.68^{b}		7.31^{b}		53.01^{a}		0.71^{a}	
4(10m S)	0-10	603.01^{e}		7.30^{e}		65.75^{bc}		0.75^{bc}	
4	10-20	400.50^{d}		5.23^{d}		50.24^{ab}		0.60^{bc}	
5(3m S)	0-10	517.11^{g}		5.41^{g}		75.37^{bc}		0.89^{bc}	
5	10-20	380.90^{e}		4.70^{f}		44.03^{bc}		0.52^{cd}	
610 m S)	0-10	481.12^{h}		5.02^{h}		64.16^{bc}		0.76^{bc}	
6	10-20	220.64^{h}		4.31^{j}		42.05^{cd}		0.50^{d}	
7(3 m N)	0-10	714.81^{c}		7.50^{c}		95.15^{cd}		1.11^{cde}	
7	10-20	325.55^{c}		5.45^{c}		40.23^{cd}		0.47^{de}	
8(3 m N)	0-10	609.32^{c}		7.50^{c}		82.78^{cd}		0.99^{cde}	
8	10-20	260.25^{d}		5.11^{e}		41.65^{cd}		0.50^{d}	
9(3 m N)	0-10	589.76^{e}		6.23^{e}		75.27^{ef}		0.88^{ef}	
9	10-20	178.34^{f}		4.40^{g}		41.06^{cd}		0.55^{d}	
10(3m N)	0-10	350.02^{i}		4.67^{i}		71.10^{fg}		0.87^{fg}	
10	10-20	60.00^{j}		0.66^{l}		44.68^{e}		0.52^{f}	
11(3m N)	0-10	100.30^{j}		1.05^{j}		69.78^{h}		0.84^{h}	
11	10-20	43.67^{k}		0.45^{m}		44.35^{e}		0.53^{h}	
12(3m S)	0-10	180.01^{j}		1.89^{j}		75.49^{h}		0.88^{h}	
12	10-20	62.54^{k}		0.65^{m}		46.57^{e}		0.54^{h}	
13(3m S)	0-10	115.72^{j}		1.21^{j}		65.30^{h}		0.78^{h}	
13	10-20	58.68^{k}		0.61^{m}		44.26^{e}		0.54^{h}	
14(10mS)	0-10	80.34^{j}		0.84^{j}		60.98^{h}		0.72^{h}	
14	10-20	35.39^{k}		0.37^{m}		41.05^{e}		0.51^{h}	
15(10mS)	0-10	48.895^{j}		0.51^{j}		65.41^{h}		0.79^{h}	
15	10-20	29.55^{k}		0.31^{m}		45.96^{e}		0.60^{h}	
16(10mN)	0-10	557.01^{f}		6.86^{j}		85.99^{cd}		1.07^{cde}	
16	10-20	501.00^{d}		6.25^{d}		40.08^{cd}		0.50^{de}	
17(10mN)	0-10	527.21^{fg}		7.50^{fg}		90.76^{de}		1.15^{de}	
17	10-20	395.42^{g}		4.15^{i}		47.21^{cd}		0.61^{de}	
18(10mN)	0-10	250.50^{gh}		2.63^{gh}		75.34^{efg}		0.93^{efg}	
18	10-20	103.14^{fg}		2.08^{h}		45.92^{d}		0.56^{e}	
19(10mN)	0-10	120.01^{i}		1.26^{i}		65.48^{g}		0.80^{g}	
19	10-20	57.82^{i}		0.61^{k}		41.23^{e}		0.48^{fg}	
20(10mN)	0-10	50.89^{j}		0.53^{j}		65.40^{h}		0.81^{h}	
20	10-20	22.90^{k}		0.24^{m}		41.01^{e}		0.50^{gh}	

a, b ,c , d ,e mean don't share the same letter are significantly different.

Appendix 2. The stat analysis for distance (1 & 2were only considered)

General Linear Model: Co versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Co, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS     F      P
DISTANCE   19  20.1971  20.1971  1.0630  2.65  0.001
Error     106  42.5947  42.5947  0.4018
Total     125  62.7918


S = 0.633906   R-Sq = 32.17%   R-Sq(adj) = 20.01%


Unusual Observations for Co

Obs       Co      Fit   SE Fit  Residual  St Resid
 38  0.60000  1.80500  0.25879  -1.20500     -2.08 R
 39  0.60000  1.80500  0.25879  -1.20500     -2.08 R
 40  7.50000  1.80500  0.25879   5.69500      9.84 R
 41  0.60000  1.80500  0.25879  -1.20500     -2.08 R
 42  0.63000  1.80500  0.25879  -1.17500     -2.03 R

R denotes an observation with a large standardized residual.


Means that do not share a letter are significantly different.
```

General Linear Model: Cr versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Cr, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS      F      P
DISTANCE   19  750.452  750.452  39.497  38.64  0.000
Error     106  108.346  108.346   1.022
Total     125  858.798


S = 1.01101   R-Sq = 87.38%   R-Sq(adj) = 85.12%
```

General Linear Model: Cu versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Cu, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS      F      P
DISTANCE   19  2751.86  2751.86  144.83  15.15  0.000
Error     106  1013.70  1013.70    9.56
Total     125  3765.55


S = 3.09244   R-Sq = 73.08%   R-Sq(adj) = 68.25%
```

General Linear Model: Mn versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Mn, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS      F      P
DISTANCE   19  4235.76  4235.76  222.93  15.74  0.000
Error     106  1501.29  1501.29   14.16
Total     125  5737.05


S = 3.76339   R-Sq = 73.83%   R-Sq(adj) = 69.14%
```

General Linear Model: Mo versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Mo, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS       F      P
DISTANCE   19  49.0754  49.0754  2.5829  118.17  0.000
Error     106   2.3169   2.3169  0.0219
Total     125  51.3923


S = 0.147845   R-Sq = 95.49%   R-Sq(adj) = 94.68%
```

General Linear Model: Ni versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Ni, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS  Adj MS      F      P
DISTANCE   19  120.6508  120.6508  6.3500  23.41  0.000
Error     106   28.7499   28.7499  0.2712
Total     125  149.4007


S = 0.520793   R-Sq = 80.76%   R-Sq(adj) = 77.31%


Unusual Observations for Ni

Obs       Ni      Fit   SE Fit  Residual  St Resid
  7  3.50000  4.63000  0.21261  -1.13000     -2.38 R
  8  6.67000  4.63000  0.21261   2.04000      4.29 R
 19  5.13000  3.40500  0.21261   1.72500      3.63 R
 20  2.12000  3.40500  0.21261  -1.28500     -2.70 R
 25  6.56000  5.09500  0.21261   1.46500      3.08 R
 33  6.49000  4.67500  0.21261   1.81500      3.82 R
 34  3.37000  4.67500  0.21261  -1.30500     -2.74 R
 35  3.02000  4.67500  0.21261  -1.65500     -3.48 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.
```

General Linear Model: Fe versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                         17, 18, 19, 20


Analysis of Variance for Fe, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS  Adj MS     F      P
DISTANCE   19  15634322  15634322  822859  1.50  0.099
Error     106  58062834  58062834  547763
Total     125  73697157
```

```
S = 740.110   R-Sq = 21.21%   R-Sq(adj) = 7.09%


Unusual Observations for Fe

Obs       Fe      Fit  SE Fit  Residual  St Resid
 76   920.40  2508.01  302.15  -1587.61     -2.35 R
 77  9051.00  2508.01  302.15   6542.99      9.68 R
 78   890.64  2508.01  302.15  -1617.37     -2.39 R

R denotes an observation with a large standardized residual.



Grouping Information Using Tukey Method and 95.0% Confidence
```

General Linear Model: Cd versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                        17, 18, 19, 20


Analysis of Variance for Cd, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS    Adj MS     F      P
DISTANCE   19  0.511976  0.511976  0.026946  3.54  0.000
Error     106  0.806717  0.806717  0.007611
Total     125  1.318693


S = 0.0872384   R-Sq = 38.82%   R-Sq(adj) = 27.86%


Grouping Information Using Tukey Method and 95.0% Confidence

Means that do not share a letter are significantly different.
```

General Linear Model: Pb versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                        17, 18, 19, 20


Analysis of Variance for Pb, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS      F      P
DISTANCE   19  8325211  8325211  438169  74.23  0.000
Error     106   625711   625711    5903
Total     125  8950921
```

```
S = 76.8305   R-Sq = 93.01%   R-Sq(adj) = 91.76%


Unusual Observations for Pb

Obs       Pb      Fit  SE Fit  Residual  St Resid
 31  501.010  350.792  31.366   150.218      2.14 R
 55  356.860  205.180  31.366   151.680      2.16 R
 57  350.320  205.180  31.366   145.140      2.07 R
 58   64.020  205.180  31.366  -141.160     -2.01 R
 59   58.780  205.180  31.366  -146.400     -2.09 R
 60   58.430  205.180  31.366  -146.750     -2.09 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.
```

General Linear Model: Zn versus DISTANCE

```
Factor    Type   Levels  Values
DISTANCE  fixed      20  1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
                        17, 18, 19, 20


Analysis of Variance for Zn, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS      F      P
DISTANCE   19  33333.4  33333.4  1754.4  69.55  0.000
Error     106   2673.7   2673.7    25.2
Total     125  36007.1


S = 5.02232   R-Sq = 92.57%   R-Sq(adj) = 91.24%


R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.
```

Sata analysis for depth (1& 2 were only considered)

General Linear Model: Co versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Co, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS     F      P
DEPTH     1   0.2171   0.2171  0.2171  0.43  0.513
Error   124  62.5747  62.5747  0.5046
Total   125  62.7918


S = 0.710376   R-Sq = 0.35%   R-Sq(adj) = 0.00%


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.
```

General Linear Model: Cr versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Cr, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS     F      P
DEPTH     1   44.215   44.215  44.215  6.73  0.011
Error   124  814.583  814.583   6.569
Total   125  858.798


S = 2.56305   R-Sq = 5.15%   R-Sq(adj) = 4.38%


R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.


Tukey Simultaneous Tests
Response Variable Cr
All Pairwise Comparisons among Levels of DEPTH
```

General Linear Model: Cu versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Cu, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS      F      P
DEPTH     1   616.28   616.28  616.28  24.27  0.000
Error   124  3149.27  3149.27   25.40
Total   125  3765.55


S = 5.03958   R-Sq = 16.37%   R-Sq(adj) = 15.69%



Means that do not share a letter are significantly different.
```

General Linear Model: Mn versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Mn, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS      F      P
DEPTH     1   835.72   835.72  835.72  21.14  0.000
Error   124  4901.33  4901.33   39.53
Total   125  5737.05


S = 6.28704   R-Sq = 14.57%   R-Sq(adj) = 13.88%
```

General Linear Model: Mo versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Mo, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS     F      P
DEPTH     1   0.6674   0.6674  0.6674  1.63  0.204
Error   124  50.7250  50.7250  0.4091
Total   125  51.3923


S = 0.639588   R-Sq = 1.30%   R-Sq(adj) = 0.50%
```

General Linear Model: Ni versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Ni, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS  Adj MS     F      P
DEPTH     1    7.124    7.124   7.124  6.21  0.014
Error   124  142.277  142.277   1.147
Total   125  149.401


S = 1.07116   R-Sq = 4.77%   R-Sq(adj) = 4.00%


Unusual Observations for Ni

Obs       Ni      Fit   SE Fit  Residual  St Resid
  8  6.67000  3.11333  0.13495   3.55667      3.35 R
 25  6.56000  3.11333  0.13495   3.44667      3.24 R
 26  5.64000  3.11333  0.13495   2.52667      2.38 R
 27  5.50000  3.11333  0.13495   2.38667      2.25 R
 32  5.34000  3.11333  0.13495   2.22667      2.10 R
 33  6.49000  3.11333  0.13495   3.37667      3.18 R

R denotes an observation with a large standardized residual.


Least Squares Means for Ni

DEPTH   Mean  SE Mean
1      3.113   0.1350
2      2.638   0.1350


Grouping Information Using Tukey Method and 95.0% Confidence


Means that do not share a letter are significantly different.


Tukey Simultaneous Tests
Response Variable Ni
All Pairwise Comparisons among Levels of DEPTH
```

General Linear Model: Fe versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Fe, using Adjusted SS for Tests
```

```
Source   DF    Seq SS    Adj SS   Adj MS     F      P
DEPTH     1   2576114   2576114  2576114  4.49  0.036
Error   124  71121043  71121043   573557
Total   125  73697157


S = 757.335   R-Sq = 3.50%   R-Sq(adj) = 2.72%


Tukey Simultaneous Tests
Response Variable Fe
All Pairwise Comparisons among Levels of DEPTH
DEPTH = 1  subtracted from:

       Difference       SE of           Adjusted
DEPTH    of Means  Difference  T-Value   P-Value
2          -286.0       134.9   -2.119    0.0361
```

General Linear Model: Cd versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2


Analysis of Variance for Cd, using Adjusted SS for Tests

Source   DF   Seq SS   Adj SS   Adj MS     F      P
DEPTH     1  0.03401  0.03401  0.03401  3.28  0.072
Error   124  1.28469  1.28469  0.01036
Total   125  1.31869


S = 0.101786   R-Sq = 2.58%   R-Sq(adj) = 1.79%


Unusual Observations for Cd

Obs        Cd       Fit    SE Fit  Residual  St Resid
  2  0.770000  0.096190  0.012824  0.673810      6.67 R
  3  0.950000  0.096190  0.012824  0.853810      8.46 R


Means that do not share a letter are significantly different.


Tukey Simultaneous Tests
Response Variable Cd
All Pairwise Comparisons among Levels of DEPTH
```

General Linear Model: Pb versus DEPTH

```
Factor  Type   Levels  Values
DEPTH   fixed       2  1, 2
```

Analysis of Variance for Pb, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DEPTH	1	378574	378574	378574	5.48	0.021
Error	124	8572348	8572348	69132		
Total	125	8950921				

S = 262.929   R-Sq = 4.23%   R-Sq(adj) = 3.46%

Grouping Information Using Tukey Method and 95.0% Confidence

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests
Response Variable Pb
All Pairwise Comparisons among Levels of DEPTH

General Linear Model: Zn versus DEPTH

Factor	Type	Levels	Values
DEPTH	fixed	2	1, 2

Analysis of Variance for Zn, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DEPTH	1	1246.1	1246.1	1246.1	4.45	0.037
Error	124	34761.0	34761.0	280.3		
Total	125	36007.1				

S = 16.7431   R-Sq = 3.46%   R-Sq(adj) = 2.68%

Unusual Observations for Zn

Obs	Zn	Fit	SE Fit	Residual	St Resid
1	73.6900	37.9254	2.1094	35.7646	2.15 R
18	68.7700	31.6357	2.1094	37.1343	2.24 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Means that do not share a letter are significantly different.

Appendix 3a. Heavy metal extraction

		extraction					
1		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.021176	0.129412	0.304118	0.318588	8.847368	0.791176
	10*20	0.014118	0.094235	0.261765	0.251059	8.001053	0.65502
2		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.017647	0.111824	0.282941	0.284824	8.424211	0.723098
	10*20	0.015882	0.103029	0.272353	0.267941	8.212632	0.689059
3		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.022353	0.136	0.293882	0.319098	8.484211	0.735059
	10*20	0.013059	0.088824	0.239529	0.262706	7.378947	0.71549
4		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.013059	0.088824	0.239529	0.262706	7.378947	0.71549
	10*20	0.014471	0.098235	0.147765	0.128118	5.263158	0.584235
5		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.010667	0.067647	0.217412	0.224118	5.442105	0.631176
	10*20	0.010353	0.061412	0.156118	0.198235	4.757895	0.521294
6		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.010588	0.123176	0.244118	0.265412	5.067368	0.633529
	10*20	0.007059	0.090118	0.181647	0.181647	2.317719	0.492353
7		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.008235	0.071176	0.182353	0.264706	7.510526	0.557647
	10*20	0.034235	0.058235	0.134353	0.222	5.480632	0.475059
8		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.012941	0.055294	0.158863	0.287176	7.464211	0.550588
	10*20	0.012549	0.05	0.120588	0.241176	5.121053	0.485059
9		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.006471	0.060588	0.156824	0.282353	6.205263	0.485294
	10*20	ND	0.058235	0.148706	0.265412	4.415789	0.478235
10		Co	Cr	Cu	Mn	Pb	Zn

	0*10	0.011176	0.054706	0.140588	0.481765	3.683684	0.376471
	10*20	0.010588	0.053176	0.124471	0.445765	0.635895	0.242235
11		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.005882	0.041176	0.288824	0.327647	0.336842	0.193529
	10*20	0.005882	0.029412	0.231333	0.235647	0.230211	0.150353
12		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.006471	0.035294	0.294235	0.288824	0.266316	0.182941
	10*20	ND	0.028824	0.192	0.180353	0.178526	0.125294
13							
	0*10	0.006275	0.034745	0.293804	0.289529	0.255158	0.173451
	10*20	ND	0.029804	0.204824	0.197843	0.157053	0.104588
14		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.006392	0.03451	0.297882	0.26749	0.258421	0.172118
	10*20	ND	0.028745	0.17898	0.164706	0.152316	0.119059
15		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.006118	0.033725	0.289804	0.28302	0.247333	0.168824
	10*20	ND	0.027922	0.20251	0.199255	0.154632	0.102078
16		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.008157	0.070745	0.175843	0.261294	5.864667	0.519373
	10*20	0.007059	0.051176	0.124902	0.207333	5.283544	0.459608
17		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.012745	0.055922	0.153059	0.276667	5.547789	0.506431
	10*20	0.012235	0.048471	0.115804	0.240824	4.164042	0.46498
18		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.00702	0.059255	0.146157	0.282118	5.315368	0.456941
	10*20	0.005765	0.05302	0.118863	0.25098	4.305158	0.395333
19		Co	Cr	Cu	Mn	Pb	Zn
	0*10	0.011216	0.051098	0.144745	0.476549	3.450982	0.357843
	10*20	0.010078	0.047176	0.124667	0.412824	1.082947	0.233569
20		Co	Cr	Cu	Mn	Pb	Zn
	0*10	ND	0.039882	0.274	0.330667	0.324	0.182431
	10*20	ND	0.028745	0.219843	0.229843	0.218316	0.146353

Appendix 3b. Heavy metal extraction

1		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.031765	11.49474	0.007216	8.847368	0.791176
	10*20	ND	0.029412	10.31579	0.001059	8.001053	0.65502

2		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.030588	10.90526	0.004137	8.424211	0.723098
	10*20	ND	0.03	10.61053	0.002598	8.212632	0.689059

3		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.014745	0.034118	16.94737	ND	8.484211	0.735059
	10*20	0.013765	0.030588	12.63158	Nd	7.378947	0.71549

4		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.013765	0.030588	12.63158	ND	7.378947	0.71549
	10*20	ND	0.037765	13.73684	ND	5.263158	0.584235

5		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.008235	0.069412	13.54737	ND	5.442105	0.631176
	10*20	0.006824	0.050471	9.368421	ND	4.757895	0.521294

6		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.015098	0.066824	13.89474	ND	5.067368	0.633529
	10*20	0.008353	0.043176	9.337895	ND	2.317719	0.492353

7		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.014706	0.029412	14.89474	0.001176	7.510526	0.557647
	10*20	0.014235	0.029294	10.53719	0.001176	5.480632	0.475059

8		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.015882	0.048235	15.81053	ND	7.464211	0.550588
	10*20	0.014706	0.046824	11.57895	ND	5.121053	0.485059

9		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.024118	0.034118	19.96526	ND	6.205263	0.485294
	10*20	0.023765	0.028824	15.78961	ND	4.415789	0.478235

10		Mo	Ni	Fe	Cd	Pb	Zn

	0*10	ND	0.034118	11.45263	0.001176	3.683684	0.376471
	10*20	ND	0.033765	8.005263	0.001059	0.635895	0.242235
11		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.023529	0.023529	14.03168	ND	0.336842	0.193529
	10*20	0.018824	0.018824	9.263684	ND	0.230211	0.150353
12		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.025294	16.8	ND	0.266316	0.182941
	10*20	ND	0.020588	9.484737	ND	0.178526	0.125294
13							
	0*10	ND	0.024471	14.68772	ND	0.255158	0.173451
	10*20	ND	0.02051	38.11242	ND	0.157053	0.104588
14		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.023843	15.33867	ND	0.258421	0.172118
	10*20	ND	0.019765	6.696737	ND	0.152316	0.119059
15		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.024471	14.37368	ND	0.247333	0.168824
	10*20	ND	0.02051	9.480632	ND	0.154632	0.102078
16		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.014824	0.029333	14.89474	ND	5.864667	0.519373
	10*20	0.01298	0.027608	10.48418	ND	5.283544	0.459608
17		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.015765	0.045961	15.73989	ND	5.547789	0.506431
	10*20	0.014	0.036941	11.44614	ND	4.164042	0.46498
18		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	0.023608	0.033098	19.88793	ND	5.315368	0.456941
	10*20	0.022627	0.028588	15.67919	ND	4.305158	0.395333
19		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.033176	11.46702	ND	3.450982	0.357843
	10*20	ND	0.032118	8.680632	ND	1.082947	0.233569
20		Mo	Ni	Fe	Cd	Pb	Zn
	0*10	ND	0.023137	14.086	ND	0.324	0.182431
	10*20	ND	0.018	9.257088	ND	0.218316	0.146353

Appendix 4. Heavy metal in Doedonea viscose leaves

REPLICATE	Co	Cr	Cu	Ni	Fe	Cd	Pb	Zn
1	0.026	0.026	0.251	0.489	27.02	0.003	0.139	2.697
2	0.026	0.024	0.25	0.49	26.59	0.003	0.147	2.813
3	0.025	0.024	0.256	0.491	27.71	0.002	0.156	2.71
mean	0.025667	0.024667	0.252333	0.49	27.10667	0.002667	0.147333	2.74

Appendix 5. Heavy metal in Doedonea viscose roots

REPLICATE	Co	Cr	Cu	Ni	Fe	Cd	Pb	Zn
1	0.0295	0.03	0.3	0.5	30.01	0.004	0.3	3.01
2	0.031	0.028	0.29	0.51	29.88	0.004	0.28	2.98
3	0.03	0.031	0.285	0.55	29.12	0.004	0.31	3.11
Mean	0.030167	0.029667	0.291667	0.52	29.67	0.004	0.296667	3.033333

Appendix 6a. Analysis of roots and shoot heavy metal `in three plants

species	cotminatio	fresh root	fresh shoot	dry root	dry shoot
M	Control soil	1.3	10.5	0.4	2
M	Control soil	1.4	10.4	0.6	1.9
M	Control soil	1.2	10.6	0.5	2.1
M	Contaminated soil	0.5	3	0.1	0.4
M	Contaminated soil	0.4	2.8	0.3	0.3
M	Contaminated soil	0.6	3.2	0.2	0.5
E	Control soil	2.6	13	0.6	4
E	Control soil	2.4	11	0.5	3.8
E	Control soil	2.8	15	0.4	4.2
E	Contaminated soil	0.9	3.2	0.3	0.5
E	Contaminated soil	0.8	3.4	0.2	0.6
E	Contaminated soil	1	3	0.1	0.4
I	Control soil	5.2	16.3	1.9	5.1
I	Control soil	5.4	16.1	1.7	5
I	Control soil	5	16.2	1.8	4.9
I	Contaminated soil	2.9	4.4	0.4	1.7
I	Contaminated soil	3	4	0.8	1.6
I	Contaminated soil	2.8	4.2	0.6	1.5

Appendix 6b. Analysis of roots and shoot heavy metal `in three plants

species	Root Cu concentration	Root Cu concentration	Shoot Cu concentration	Root Cr concentration	Shoot Cr concentration
M	Control soil	67	29	11	2.1
M	Control soil	68	27	12	2
M	Control soil	70	28	10.5	2.2
M	Contaminated soil	73	37	24	9.2
M	Contaminated soil	71	38	26	9
M	Contaminated soil	69	36	22	9.5
E	Control soil	87	12.4	37.5	3.5
E	Control soil	85	12.3	35	3.6
E	Control soil	80	12.5	33	3.4
E	Contaminated soil	81	18	133.9	24
E	Contaminated soil	80	20	134	25
E	Contaminated soil	78	17	133.8	23
I	Control soil	76	6.2	3.2	0.2
I	Control soil	70	7	3.3	0.1
I		69	6	3.1	0.3
I	Contaminated soil	80	11	25.5	2.2
I	Contaminated soil	79	9	26	2.3
I	Contaminated soil	65	10	24	2.1

Appendix 6c. Analysis of roots and shoot heavy metal `in three plants

species	cotminatio	Root Pb concentration	Shoot Pb concentration	Root Zn concentration	Shoot Zn concentration
M	Control soil	477	50.3	36.1	25.2
M	Control soil	476	50.4	34	27
M	Control soil	478	50.5	35	28
M	Contaminated soil	1058	440	71	49
M	Contaminated soil	1060	436	68	50
M	Contaminated soil	1056	438	69	48
E	Control soil	130	49.7	26.6	14.5
E	Control soil	131	49.6	25	14
E	Control soil	132	49.8	27	16
E	Contaminated soil	989	548	86	47
E	Contaminated soil	990	549	84	43
E	Contaminated soil	988	547	85	48
I	Control soil	55.7	6.4	33	21
I	Control soil	55.8	6.6	31	20
I	Control soil	55.9	6.5	32	22
I	Contaminated soil	485	57.6	56	41.5
I	Contaminated soil	490	57.8	58	39.5
I	Contaminated soil	480	57.4	54	40
species	cotminatio				

Appendix 6d Analysis of roots and shoot heavy metal `in three plants

General Linear Model: fresh root versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for fresh root, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS   Adj MS       F      P
species              2  31.8700  31.8700  15.9350  796.75  0.000
cotminatio           1  11.5200  11.5200  11.5200  576.00  0.000
species*cotminatio   2   1.7100   1.7100   0.8550   42.75  0.000
Error               12   0.2400   0.2400   0.0200
Total               17  45.3400

Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
I        6   4.0  A
E        6   1.7    B
M        6   0.9      C

Means that do not share a letter are significantly different.
```

General Linear Model: fresh shoot versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for fresh shoot, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS   Adj MS       F      P
species              2   36.270   36.270   18.135   26.28  0.000
cotminatio           1  429.245  429.245  429.245  622.09  0.000
species*cotminatio   2   15.190   15.190    7.595   11.01  0.002
Error               12    8.280    8.280    0.690
Total               17  488.985


S = 0.830662   R-Sq = 98.31%   R-Sq(adj) = 97.60%

Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
I        6  10.2  A
E        6   8.1    B
M        6   6.7      C

Means that do not share a letter are significantly different.
```

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio	N	Mean	Grouping
Uncontaminated	9	13.2	A
Contaminated soil	9	3.5	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping
I	Uncontaminated	3	16.2	A
E	Uncontaminated	3	13.0	B
M	Uncontaminated	3	10.5	C
I	Contaminated soil	3	4.2	D
E	Contaminated soil	3	3.2	D
M	Contaminated soil	3	3.0	D

Means that do not share a letter are significantly different.

General Linear Model: dry root versus species, contamination

Factor	Type	Levels	Values
species	fixed	3	E, I, M
cotminatio	fixed	2	Contaminated soil, Uncontaminated

Analysis of Variance for dry root, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
species	2	2.8900	2.8900	1.4450	96.33	0.000
cotminatio	1	1.6200	1.6200	1.6200	108.00	0.000
species*cotminatio	2	0.8100	0.8100	0.4050	27.00	0.000
Error	12	0.1800	0.1800	0.0150		
Total	17	5.5000				

Grouping Information Using Tukey Method and 95.0% Confidence

species	N	Mean	Grouping
I	6	1.2	A
M	6	0.4	B
E	6	0.3	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio	N	Mean	Grouping
Uncontaminated	9	0.9	A
Contaminated soil	9	0.3	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping

```
I        Uncontaminated     3   1.8  A
I        Contaminated soil  3   0.6    B
M        Uncontaminated     3   0.5    B C
E        Uncontaminated     3   0.5    B C
M        Contaminated soil  3   0.2      C
E        Contaminated soil  3   0.2      C

Means that do not share a letter are significantly different.
```

General Linear Model: dry shoot versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for dry shoot, using Adjusted SS for Tests

Source              DF  Seq SS  Adj SS  Adj MS        F      P
species              2  13.230  13.230   6.615   441.00  0.000
cotminatio           1  36.125  36.125  36.125  2408.33  0.000
species*cotminatio   2   3.430   3.430   1.715   114.33  0.000
Error               12   0.180   0.180   0.015
Total               17  52.965


S = 0.122474   R-Sq = 99.66%   R-Sq(adj) = 99.52%


Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
I        6   3.3  A
E        6   2.3    B
M        6   1.2      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio         N  Mean  Grouping
Uncontaminated     9   3.7  A
Contaminated soil  9   0.8    B

Means that do not share a letter are significantly different.


Tukey Simultaneous Tests
Response Variable dry shoot
All Pairwise Comparisons among Levels of contamination
cotminatio = Contaminated soil  subtracted from:

                Difference       SE of           Adjusted
cotmination        of Means  Difference  T-Value   P-Value
Uncontaminated       2.833     0.05774    49.07    0.0000
```

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping
I	Uncontaminated	3	5.0	A
E	Uncontaminated	3	4.0	B
M	Uncontaminated	3	2.0	C
I	Contaminated soil	3	1.6	D
E	Contaminated soil	3	0.5	E
M	Contaminated soil	3	0.4	E

Means that do not share a letter are significantly different.

General Linear Model: Root Pb concentration versus species, contamination

Factor	Type	Levels	Values
species	fixed	3	E, I, M
cotminatio	fixed	2	Contaminated soil, Uncontaminated

Analysis of Variance for Root Pb concentration, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
species	2	748066	748066	374033	70109.24	0.000
cotminatio	1	1745086	1745086	1745086	327101.33	0.000
species*cotminatio	2	141821	141821	70910	13291.55	0.000
Error	12	64	64	5		
Total	17	2635036				

S = 2.30976   R-Sq = 100.00%   R-Sq(adj) = 100.00%

Grouping Information Using Tukey Method and 95.0% Confidence

species	N	Mean	Grouping
M	6	767.5	A
E	6	560.0	B
I	6	270.4	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio	N	Mean	Grouping
Contaminated soil	9	844.0	A
Uncontaminated	9	221.3	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping
M	Contaminated soil	3	1058.0	A
E	Contaminated soil	3	989.0	B
I	Contaminated soil	3	485.0	C

```
M        Uncontaminated     3   477.0        D
E        Uncontaminated     3   131.0          E
I        Uncontaminated     3    55.8            F
```

Means that do not share a letter are significantly different.

General Linear Model: Shoot Pb concentration versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated
```

Analysis of Variance for Shoot Pb concentration, using Adjusted SS for Tests

```
Source              DF  Seq SS  Adj SS  Adj MS          F      P
species              2  238353  238353  119176  141037.26  0.000
cotminatio           1  438985  438985  438985  519508.28  0.000
species*cotminatio   2  162737  162737   81369   96294.25  0.000
Error               12      10      10       1
Total               17  840085
```

Grouping Information Using Tukey Method and 95.0% Confidence

```
species  N   Mean  Grouping
E        6  298.9  A
M        6  244.2    B
I        6   32.0      C
```

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

```
cotmination         N   Mean  Grouping
Contaminated soil  9  347.9  A
Uncontaminated     9   35.5    B
```

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

```
species  cotminatio         N   Mean  Grouping
E        Contaminated soil  3  548.0  A
M        Contaminated soil  3  438.0    B
I        Contaminated soil  3   57.6      C
M        Uncontaminated     3   50.4        D
E        Uncontaminated     3   49.7        D
I        Uncontaminated     3    6.5          E
```

Means that do not share a letter are significantly different.

General Linear Model: Root Zn concentration versus species, cotminatio

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for Root Zn concentration, using Adjusted SS for Tests

Source              DF  Seq SS  Adj SS  Adj MS        F      P
species              2   426.4   426.4   213.2   121.17  0.000
cotminatio           1  6856.2  6856.2  6856.2  3896.80  0.000
species*cotmination   2   958.7   958.7   479.3   272.44  0.000
Error               12    21.1    21.1     1.8
Total               17  8262.4


Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
E        6  55.6  A
M        6  52.2    B
I        6  44.0      C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio         N  Mean  Grouping
Contaminated soil  9  70.1  A
Uncontaminated     9  31.1    B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species  cotminatio         N  Mean  Grouping
E        Contaminated soil  3  85.0  A
M        Contaminated soil  3  69.3    B
I        Contaminated soil  3  56.0      C
M        Uncontaminated     3  35.0        D
I        Uncontaminated     3  32.0        D
E        Uncontaminated     3  26.2          E

Means that do not share a letter are significantly different.
```

General Linear Model: Shoot Zn concentration versus species, cotminatio

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for Shoot Zn concentration, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS   Adj MS        F      P
species              2   214.81   214.81   107.41    48.89  0.000
```

```
cotmination           1  2647.49  2647.49  2647.49  1205.23  0.000
species*cotmination   2   113.92   113.92    56.96    25.93  0.000
Error                12    26.36    26.36     2.20
Total                17  3002.59


Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
M        6  37.9  A
I        6  30.7    B
E        6  30.4    B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotmination         N  Mean  Grouping
Contaminated soil  9  45.1  A
Uncontaminated     9  20.9    B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species  cotmination         N  Mean  Grouping
M        Contaminated soil  3  49.0  A
E        Contaminated soil  3  46.0  A
I        Contaminated soil  3  40.3    B
M        Uncontaminated     3  26.7      C
I        Uncontaminated     3  21.0        D
E        Uncontaminated     3  14.8          E

Means that do not share a letter are significantly different.
```

General Linear Model: Root Cu concentration versus species, cotminatio

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for Root Cu concentration, using Adjusted SS for Tests

Source              DF  Seq SS  Adj SS  Adj MS      F      P
species              2  470.78  470.78  235.39  13.28  0.001
cotmination          1    0.89    0.89    0.89   0.05  0.827
species*cotmination  2   51.44   51.44   25.72   1.45  0.273
Error               12  212.67  212.67   17.72
Total               17  735.78
Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
E        6  81.8  A
I        6  73.2    B
M        6  69.7    B
```

Grouping Information Using Tukey Method and 95.0% Confidence

cotmination	N	Mean	Grouping
Contaminated soil	9	75.1	A
Uncontaminated	9	74.7	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping
E	Uncontaminated	3	84.0	A
E	Contaminated soil	3	79.7	A B
I	Contaminated soil	3	74.7	A B
I	Uncontaminated	3	71.7	B
M	Contaminated soil	3	71.0	B
M	Uncontaminated	3	68.3	B

Means that do not share a letter are significantly different.

General Linear Model: Shoot Cu concentration versus species, contamination

Factor	Type	Levels	Values
species	fixed	3	E, I, M
cotminatio	fixed	2	Contaminated soil, Uncontaminated

Analysis of Variance for Shoot Cu concentration, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
species	2	1870.80	1870.80	935.40	998.06	0.000
cotminatio	1	171.74	171.74	171.74	183.25	0.000
species*cotminatio	2	22.00	22.00	11.00	11.74	0.001
Error	12	11.25	11.25	0.94		
Total	17	2075.80				

Grouping Information Using Tukey Method and 95.0% Confidence

species	N	Mean	Grouping
M	6	32.5	A
E	6	15.4	B
I	6	8.2	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio	N	Mean	Grouping
Contaminated soil	9	21.8	A
Uncontaminated	9	15.6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

```
species  cotminatio         N  Mean  Grouping
M        Contaminated soil  3  37.0  A
M        Uncontaminated     3  28.0    B
E        Contaminated soil  3  18.3      C
E        Uncontaminated     3  12.4        D
I        Contaminated soil  3  10.0        D
I        Uncontaminated     3   6.4          E

Means that do not share a letter are significantly different.
```

General Linear Model: Root Cr concentration versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for Root Cr concentration, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS  Adj MS        F      P
species              2  18886.0  18886.0  9443.0  5260.72  0.000
cotminatio           1   8915.6   8915.6  8915.6  4966.89  0.000
species*cotminatio   2   6677.7   6677.7  3338.8  1860.08  0.000
Error               12     21.5     21.5     1.8
Total               17  34500.8

Grouping Information Using Tukey Method and 95.0% Confidence

species  N  Mean  Grouping
E        6  84.5  A
M        6  17.6    B
I        6  14.2      C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio         N  Mean  Grouping
Contaminated soil  9  61.0  A
Uncontaminated     9  16.5    B

Means that do not share a letter are significantly different.
```

General Linear Model: Shoot Cr concentration versus species, contamination

```
Factor      Type   Levels  Values
species     fixed       3  E, I, M
cotminatio  fixed       2  Contaminated soil, Uncontaminated


Analysis of Variance for Shoot Cr concentration, using Adjusted SS for Tests

Source              DF   Seq SS  Adj SS  Adj MS        F      P
species              2   485.59  485.59  242.79  1320.33  0.000
```

cotminatio	1	439.07	439.07	439.07	2387.68	0.000
species*cotminatio	2	273.63	273.63	136.82	744.02	0.000
Error	12	2.21	2.21	0.18		
Total	17	1200.50				

Grouping Information Using Tukey Method and 95.0% Confidence

species	N	Mean	Grouping
E	6	13.8	A
M	6	5.7	B
I	6	1.2	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

cotminatio	N	Mean	Grouping
Contaminated soil	9	11.8	A
Uncontaminated	9	1.9	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

species	cotminatio	N	Mean	Grouping
E	Contaminated soil	3	24.0	A
M	Contaminated soil	3	9.2	B
E	Uncontaminated	3	3.5	C
I	Contaminated soil	3	2.2	D
M	Uncontaminated	3	2.1	D
I	Uncontaminated	3	0.2	E

Means that do not share a letter are significantly different

Appendix 7 The composition of Hoagland's solution

1. $Ca(NO_3)_2.4H_2O$ 236.1 $g.l^{-1}$
2. KNO3 101.1 $g.l^{-1}$
3. KH2PO4 136.1 $g.l^{-1}$
4. MgSO4.7H2O 246.5 $g.l^{-1}$
5. Trace elements (made up to 1 litre): H_3BO_3 2.8 g; $MnCl_2.4H_2O$ 1.8 g; $ZnSO_4.7H_2O$ 0.2 g; $CuSO_4.5H_2O$ 0.1 g; and $NaMoO_4$ 0.025 g.
6. FeEDTA (10.4 g EDTA.2Na; 7.8 g $FeSO_4.7H_2O$; 56.1 g KOH)

Make up 1 L of KOH, adjust pH to ~5.5 using H2SO4, and then add EDTA.2Na and $FeSO_4.7H_2O$.

To make 1 L Hoagland's solution from these stocks were add:

7 ml $Ca(NO_3)_2$ stock; 5 ml KNO_3; 2 ml KH_2PO_4; 2 ml $MgSO_4$; 1 ml Trace elements; 1 ml FeEDTA to 1 L water.

Appendix 8 Fresh and dry wt roots and shoots of Eucalyptus plant

		dry wt				Fresh wt	
		Shoot	**Root**			**Shoot**	**Root**
Contarl	1	0.5	0.2	**Contarl**	1	3.6	0.9
	2	0.6	0.3		2	3.7	1
	3	0.4	0.1		3	3.5	0.8
Compast	C5 1	1.2	0.3	**compast**	C5 1	4.8	1.6
	2	1.3	0.2		2	4.7	1.7
	3	1.1	0.4		3	4.9	1.5
	C10 1	1.8	0.5		C10 1	5.6	2.5
	2	2	0.6		2	5.7	2.6
	3	1.6	0.4		3	5.6	2.4
	C25 1	2.5	0.8		C25 1	8.7	2.9
	2	2.6	1		2	8.8	3
	3	2.4	0.6		3	8.6	2.8
EDTA	E5 1	0.7	0.16	**EDTA**	E5 1	3.6	0.9
	2	0.8	0.17		2	3.5	1
	3	0.6	0.15		3	3.7	0.8
	E10 1	0.7	0.07		E10 1	3.1	0.7
	2	0.8	0.08		2	3.2	0.8
	3	0.6	0.06		3	3	0.6
	E15 1	0.6	0.06		E15 1	2.9	0.5
	2	0.5	0.07		2	2.8	0.6
	3	0.7	0.05		3	3	0.4
Hol sou	H25 1	1.7	0.5	**Hol sou**	H25 1	6.6	2.3
	2	1.8	0.6		2	6.7	2.4
	3	1.6	0.4		3	6.5	2.2
	H50 1	2.2	0.6		H50 1	8.5	2.7
	2	2.3	0.7		2	8.6	2.8
	3	2.1	0.5		3	8.4	2.6
	H100 1	2.8	0.9		H100 1	10.4	4.3
	2	3	1		2	10.5	4.2
	3	2.6	0.8		3	10.3	4.4
Bacteri	B 1	2.4	1.2	**Bacteri**	B 1	9.8	3.1
	2	2.5	1.3		2	10	3
	3	2.3	1.1		3	9.6	3.2
mixed	Mix 1	3.1	1.5	**mixed**	Mix 1	13.4	4.5
	2	3.2	1.6		2	13.5	4.6
	3	3	1.4		3	13.3	4.4

Appendix 9 Pb concentration roots and shoots of Eucalyptus plant with amendment

		Shoot	Root
	1	544	986
Contarl	2	545	988
	3	543	984
	1	548	994
Compast C5	2	550	995
	3	546	993
	1	535	1023
C10	2	536	1025
	3	534	1020
	1	533	1018
C25	2	534	1020
	3	532	1016
	1	575	1025
EDTA E5	2	570	1027
	3	580	1023
	1	655	1054
E10	2	656	1055
	3	654	1053
	1	705	1065
E15	2	700	1066
	3	710	1064
	1	550	1011
Hol sou H25	2	548	1012
	3	552	1010
	1	557	1019
H50	2	558	1020
	3	556	1018
	1	565	1025
H100	2	566	1030
	3	564	1020
	1	695	1073
Bacteri B	2	696	1074
	3	964	1072
	1	645	1064
Mixed Mix	2	646	1065
	3	644	1063

Appendix 10 Physico -chemical of Pb unplanted contaminated soil with amendment after one month

Amendments	pH	CEC	EC	OM	N	P	Available Pb
			mmhos cm^{-1}	(%)	mg kg^{-1}		mg kg-1
Composite (C)							
0%	7.71	8.09	1.6	0.09	0.14	0.7	8.8 ± 0.1
5%	7.81	8.21	2.12	1.15	0.52	0.75	9.6 ± 0.15
10%	8.1	8.28	2.98	2.35	0.92	0.81	10.2 ± 0.25
25%	8.2	8.33	3.15	4.54	2.31	0.98	12.6 ± 0.47
EDTA (B)							
0 mmol	7.71	8.09	1.6	0.09	0.14	0.7	8.8 ± 0.1
5 mmol	7.68	8.11	2.05	0.09	0.13	0.72	14.2 ± 0.86
10 mmol	7.64	8.15	2.51	0.11	0.13	0.81	16.8 ± 0.30
15 mmol	7.6	8.25	2.98	0.12	0.15	0.83	17.6 ± 0.68
Hoagland solution (H)							
0%	7.71	8.09	1.6	0.09	0.14	0.7	8.8 ± 0.1
25%	6.54	8.25	1.82	0.16	0.19	0.81	9.5 ± 0.20
50%	6.4	8.51	2.34	0.25	0.25	0.93	11.6 ± 0.74
100%	6.05	8.95	2.87	0.37	0.48	1.52	13.1 ± 0.27
Bacterial inoculum's (B)	6.6	8.3	1.68	0.15	0.14	0.9	15.4 ± 0.66
Mixed amendments (5%C+ 5 mmol E+ 100% H+B)	6.9	9.05	2.35	0.2	0.55	2.25	17.2 ± 0.98

Appendix 11a. cacl2 extractable metal (pb mg/kg dry soil)

	Amendments		rep1 2	rep 2	rep3
compast	control		8.8	8.9	8.7
		5%	8.9	9.5	9
		10%	9.2	9.5	10
		25%	7.2	7.5	8.2
	EDTA				
	5 mmol		16.3	16	15.6
	10 mmol		18.2	17.8	19
	15 mmol		25	22	23
	Hogland				
		25%	9.1	8.8	9.5
		50%	10.1	10.5	10.4
		100%	10.3	10.1	9.9
	Bacterial		11.5	11.1	12.3
	Mix		14.5	15	15.9

Appendix 11b. Available Pb

	after one month			
		rep1	rep2	rep3
Compast	control	8.7	8.8	8.9
	5%	9.5	9.8	9.7
	10%	10.2	10.5	10.7
	25%	12.1	12.8	13
	EDTA			
	5 mmol	14.1	13.5	15.2
	10 mmol	16.5	16.9	17.1
	15 mmol	17.91	18.2	16.9
	Hogland			
	25%	9.58	9.23	9.6
	50%	11.89	10.75	12.15
	100%	13.45	12.91	13.1
	Bacterial	14.95	16.2	15.2
	Mix	17.61	18.05	16.17

Appendix 12. Physico-chemical characteristics of Pb unplanted contaminated soil with different amendment after one month in pot experiment

Amendments	**pH**	**CEC**	**EC**	**OM**	**N**	**P**	**Available Pb**
			mmhos cm^{-1}	(%)	mg kg^{-1}		mg kg^{-1}
Composite (C)							
0%	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
5%	7.81	8.21	2.12	1.15	0.52	0.75	9.6 ± 0.15
10%	8.10	8.28	2.98	2.35	0.92	0.81	10.2 ± 0.25
25%	8.20	8.33	3.15	4.54	2.31	0.98	12.6 ± 0.47
EDTA (B)							
0 mmol	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
5 mmol	7.68	8.11	2.05	0.09	0.13	0.72	14.2 ± 0.86
10 mmol	7.64	8.15	2.51	0.11	0.13	0.81	16.8 ± 0.30
15 mmol	7.60	8.25	2.98	0.12	0.15	0.83	17.6 ± 0.68
Hoagland solution (H)							
0%	7.71	8.09	1.60	0.09	0.14	0.70	8.8 ± 0.1
25%	6.54	8.25	1.82	0.16	0.19	0.81	9.5 ± 0.20
50%	6.40	8.51	2.34	0.25	0.25	0.93	11.6 ± 0.74
100%	6.05	8.95	2.87	0.37	0.48	1.52	13.1 ± 0.27
Bacterial inoculum's (B)	6.6	8.3	1.68	0.15	0.14	0.90	15.4 ± 0.66
Mixed amendments (5%C+ 5 mmol E+ 100% H+B)	6.9	9.05	2.35	0.20	0.55	2.25	17.2 ± 0.98

Appendix 13. pb concentration availability with adding amendments (pb mg/kg dry soil)

Control	8.8	8.9	8.7	8.8	0.10
5%	8.9	9.5	9	9.13	0.32
10%	9.2	9.5	10	9.56	0.40
25%	7.2	7.5	8.2	7.63	0.51
EDTA					
5 mmol	16.3	16	15.6	15.96	0.35
10 mmol	18.2	17.8	19	18.33	0.61
15 mmol	25	22	23	23.33	1.52
Hogland					
25%	9.1	8.8	9.5	9.133	0.35
50%	10.1	10.5	10.4	10.33	0.20
100%	10.3	10.1	9.9	10.1	0.20
Bacterial	11.5	11.1	12.3	11.63	0.61
Mix	14.5	15	15.9	15.133	0.70
	after one month				
control	compost			Average	
5%	9.5	9.8	9.7	9.66	0.15
10%	10.2	10.5	10.7	10.46	0.25
25%	12.1	12.8	13	12.63	0.47
EDTA				average	
5 mmol	14.1	13.5	15.2	14.2	0.86
10 mmol	16.5	16.9	17.1	16.8	0.30
15 mmol	17.91	18.2	16.9	17.6	0.68
Hogland				average	
25%	9.58	9.23	9.6	9.47	0.20
50%	11.89	10.75	12.15	11.59	0.74
100%	13.45	12.91	13.1	13.1	0.272
Bacterial	14.95	16.2	15.2	15.4	0.661438
Mix	17.61	18.05	16.17	17.2	0.983328

Appendix 14. Analysis of Variance for Pb concentration Shoot, using Adjusted SS for Tests

Source DF Seq SS Adj SS Adj MS F P

Treatment 11 213157 213157 19378 9.65 0.000

Error 24 48192 48192 2008

Total 35 261349

Grouping Information Using Tukey Method and 95.0% Confidence

Treatment N Mean Grouping

Bacteria 3 785.0 A

ETDA15 3 705.0 A B

EDTA10 3 655.0 A B C

mixed 3 645.0 B C

ETDA5 3 575.0 B C

H100 3 565.0 C

H50 3 557.0 C

H25 3 550.0 C

C5 3 548.0 C

Contarl 3 544.0 C

C10 3 535.0 C

C25 3 533.0 C

Analysis of Variance for Pb CONCENTRATION/Root, using Adjusted SS for Tests

Source DF Seq SS Adj SS Adj MS F P

Treatment 11 26300.6 26300.6 2391.0 570.03 0.000

Error 24 100.7 100.7 4.2

Total 35 26401.2

Grouping (Pb)Information Using Tukey Method and 95.0% Confidence

Treatment	N	Mean	Grouping
Bacteria	3	1073.0	A
ETDA15	3	1065.0	B
mixed	3	1064.0	B
EDTA10	3	1054.0	C
H100	3	1025.0	D
ETDA5	3	1025.0	D
C10	3	1022.7	D E
H50	3	1019.0	D E
C25	3	1018.0	E
H25	3	1011.0	F
C5	3	994.0	G
Contarl	3	986.0	H

Analysis of Variance for Shoot Fresh Weight using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	11	380.323	380.323	34.575	2894.64	0.000
Error	24	0.287	0.287	0.012		
Total	35	380.610				

Grouping Information for Shoot Fresh Weight Using Tukey Method and 95.0% Confidence

Treatment	N	Mean	Grouping
mixed	3	13.4	A
H100	3	10.4	B
Bacteria	3	9.8	C
C25	3	8.7	D

H50 3 8.5 D

H25 3 6.6 E

C10 3 5.6 F

C5 3 4.8 G

ETDA5 3 3.6 H

Control 3 3.6 H

EDTA10 3 3.1 I

EDTA15 3 2.9 I

Analysis of Variance for Root Fresh Weight using Adjusted SS for Tests

Source DF Seq SS Adj SS Adj MS F P

Treatment 11 60.6275 60.6275 5.5116 551.16 0.000

Error 24 0.2400 0.2400 0.0100

Total 35 60.8675

Grouping Information for Root Fresh Weight Using Tukey Method and 95.0% Confidence

Treatment N Mean Grouping

mixed 3 4.5 A

H100 3 4.3 A

Bacteria 3 3.1 B

C25 3 2.9 B C

H50 3 2.7 C D

C10 3 2.5 D E

H25 3 2.3 E

C5 3 1.6 F

ETDA5 3 0.9 G

Contarl 3 0.9 G

EDTA10 3 0.7 G H

ETDA15 3 0.5 H

Analysis of Variance for Shoot Dry Weight using Adjusted SS for Tests

Source DF Seq SS Adj SS Adj MS F P

Treatment 11 28.3700 28.3700 2.5791 171.94 0.000

Error 24 0.3600 0.3600 0.0150

Total 35 28.7300

Grouping Information for Shoot Dry Weight Using Tukey Method and 95.0% Confidence

Treatment N Mean Grouping

mixed 3 3.1 A

H100 3 2.8 A B

C25 3 2.5 B C

Bacteria 3 2.4 C

H50 3 2.2 C

C10 3 1.8 D

H25 3 1.7 D

C5 3 1.2 E

EDTA10 3 0.7 F

EDTA5 3 0.7 F

EDTA15 3 0.6 F

Contarl 3 0.5 F

Analysis of Variance for Root Dry Weight using Adjusted SS for Tests

Source DF Seq SS Adj SS Adj MS F P

Treatment 11 6.96628 6.96628 0.63330 63.17 0.000

Error	24	0.24060	0.24060	0.01003
Total	35	7.20688		

Grouping Information for Root Dry Weight Using Tukey Method and 95.0% Confidence

Treatment	N	Mean	Grouping
mixed	3	1.5	A
Bacteria	3	1.2	B
H100	3	0.9	C
C25	3	0.8	C D
H50	3	0.6	D E
H25	3	0.5	E F
C10	3	0.5	E F
C5	3	0.3	F G
Contarl	3	0.2	G
ETDA5	3	0.2	G
EDTA10	3	0.1	G
ETDA15	3	0.1	G